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OBSERVATIONS ON THE EFFECT OF PROGESTERONE ON CARCINOMA OF THE CERVIX[1,2]

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The usefulness of hormonal therapy in cancer of the breast and prostate (*1, 2*) suggested the possible effectiveness of steroid hormones in altering the clinical manifestations of cancer of the cervix uteri. In selecting progesterone as a steroid hormone for clinical trial, we were influenced by certain prior experimental findings with this substance. A lesion entirely comparable to cervical cancer had not been experimentally produced in the primate, but Overholser and Allen (*3*) and several other observers (*4, 5*) had shown that prolonged estrogen stimulation in the monkey produced a marked squamous metaplasia of the cervical glandular epithelium. Moreover, Hisaw and Lendrum (*4*) had shown that such estrogen-induced metaplasia could be entirely prevented by the simultaneous administration of progesterone. Thus, what had been interpreted by many as a precancerous lesion in the cervix could be markedly altered by progesterone.

Lipschutz, Murillo, and Vargas (*6*) demonstrated that estrogen administration would lead to the formation of uterine fibroids in the guinea pig. They also observed that progesterone given along with estrogen would prevent the formation of such estrogen-induced fibromyomas. They referred to this effect as the "anti-tumorigenic" action of progesterone.

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Goodman (*7*) reported a decrease in size of fibroid tumors in 7 patients treated with progesterone. Segaloff, Weed, and Parson (*8*) observed no radiographically demonstrable change in the size of fibroid tumors in 3 cases.

Gardner (*9*) has shown that the development of cervical cancer in certain strains of mice is dependent upon estrogen administration. Hertz, Larsen, and Tullner (*10*) observed that progesterone could quantitatively suppress active tissue proliferation in the genital tract of the estrogen-treated bird.

In view of these experimental findings indicating an antitumorigenic and growth-suppressant action of progesterone, a clinical study of the effect of progesterone [3] on carcinoma of the cervix was undertaken.

MATERIALS AND METHODS

Seventeen patients were selected from the Gynecology Clinic of the George Washington University Cancer Service. Their clinical status was determined at the time of their entrance into the study in accordance with the League of Nations' classification for cervical cancer (*11*). All diagnoses were confirmed by biopsies that were unequivocally interpreted as indicating the presence of malignancy. Only patients who presented clearly visible lesions were selected for this study in order that periodic photographs would permit objective appraisal of the progress of the visible portion of the carcinomatous process.

All of these patients were examined carefully each week. Since no lesion was observed to progress during the period of study, the delay preceding definitive surgical treatment was considered to be in keeping with optimum clinical management of each case.

All patients were given 250 mg. of progesterone in 5 cc. of oil intramuscularly daily for varying periods as indicated in table 1.

The following observations were made on all patients just prior to and at frequent intervals during the course of progesterone administration:

1. Photographs of the cervix taken with the aid of a Coreco camera under conditions of fixed illumination and magnification.

2. Daily urinary excretion of sodium pregnandiol glycuronidate as determined by the method of Somerville *et al.* (*12*).

3. Weekly pelvic examinations noting the visible and palpable changes in the cervix and parametria, with special emphasis on the color and size of the lesion, its mobility and friability, and the presence or absence of active bleeding.

4. Weekly appraisal of the patient's general clinical status with respect to (a) vaginal bleeding or discharge, (b) pain, (c) feeling of well being, (d) appetite, (e) urinary function, (f) bowel function, (g) body weight, and (h) possible local or systemic effects of the progesterone.

Immediately after the completion of the progesterone study, 14 of these

[3] Progesterone used in these studies was provided by the Schering Corporation through the courtesy of Dr. E. L. Henderson.

patients were afforded definitive surgical management as indicated.[4] One patient who presented far advanced disease (#2) and was considered not to be amenable to any other form of therapy was continued on progesterone until she died. One patient (#1) discontinued attendance at the clinic several months following her 153-day course of progesterone.

The age, color, clinical classification, duration of progesterone treatment, and other pertinent features of each case are set forth in table 1.

RESULTS AND DISCUSSION

In such a widely varying and yet limited body of clinical material it is difficult to make general observations. Nevertheless, certain changes in the clinical and morphological features of the lesions in the progesterone treated patients occurred with sufficient frequency to warrant description.

In 11 of the 17 treated patients, visible and palpable evidence of regressive alteration of the tumor mass could be demonstrated. This consisted of (a) distinct reduction in size of the visible portion of the cancer as well as reduction of the palpable extent of the mass, (b) reduction in vascularity and friability of the visible lesion with a clearly demonstrable epithelization of previously raw surfaces, and (c) markedly increased pliability of the previously rigid and infiltrated parametria. Figures 1 and 2 (case #16) illustrate the reduction in size of the largest lesion we have studied. Figures 3 and 4 illustrate the regression of an early lesion under the influence of progesterone in case #11. Figures 5 and 6 (case #13 represent a less marked but more frequently observed type of regressive change.

In 10 cases there was associated with this type of gross change a reduction in, or complete cessation of, vaginal bleeding and discharge.

Routine histopathological study of the biopsy and surgical specimens has not indicated the morphological basis for the observed changes. A subsequent report will present a detailed histopathological study of serial blocks of the material from all of these cases.

Only one of the 17 patients (#1) showed active progression of the carcinomatous process while under progesterone administration. The 6 patients whose lesions failed to show clearly demonstrable regressive changes showed minor alterations in size and vascularity of insufficient degree to be convincing to all clinical observers concerned. Nevertheless, none of the lesions under study appeared to be accelerated by progesterone.

Only one untoward side-reaction was observed. This was in a patient who proved allergic to the vegetable oil used as a vehicle for the progesterone. Injections were discontinued after 3 days and this patient is therefore not included in the present series. Otherwise the progesterone was well tolerated in all respects. However, the necessity for daily administration of 5 cc. of oil intramuscularly in some cases led to difficulty in varying the site of injection and this made the patient somewhat uncomfortable. It is noteworthy that 3 patients who were menstruating

[4] We will subsequently present a detailed report on the surgical aspects of these studies.

TABLE 1.—*Cancer of cervix under progesterone administration*

Case No. age and color	Description[1]	Biopsy	Previous treatment	Progesterone administered	Urinary pregnandiol recovery	Clinical response	Surgery	Surgical specimen
1 77 C	Recurrent stump Stage III	1947 Epid. Ca., gr 3 March, 1949 Residual Ca. August, 1949 Epid. Ca. gr 2	1925 Subtotal 1947—4 Full X-ray and Radium	38.25 gm. in 153 days	4%	Discharge decreased Extension delayed 3 mo.	None	None
2 43 W	Recurrence 4 months after Wertheim Recurrent Stage IV	August, 1949 Epid. Ca., gr 3	1948-49 Radium Jan. 1949 X-ray May 1949 Wertheim	34.5 gm. in 138 days	10%	Bleeding decreased Softening Leg pain lessened	None	None Autopsy:— Extension to femoral plexus.
3 65 W	Ulcerating Stage II	Epid. Ca., gr 2	None	16.25 gm. in 65 days	Not done	Cessation of bleeding Epithelization	Radical with nodes	Epid, Ca. gr 2 Atrophy-endometrium.
4 56 W	Bilat. ooph. 1926 Stage III	Epid. Ca., gr 2	None	28.85 gm. in 113 days	10%	Bleeding ceased Relaxation Epithelization	Radical with nodes Cystectomy Bilat. uret transplant	Epid. Ca., gr 3 with extension to corpus. 3 of 18 nodes positive. Atrophy-endometrium.
5 54 W	Asymptomatic erosion Stage I	Epid. Ca., gr 3	None	1.5 gm. in 10 days	13%	Epithelization Softening	Radical with nodes	Epid. Ca.—*in situ*
6 36 W	Ovarian cyst Erosion Late Stage I	Epid. Ca., gr 2	None	2.0 gm. in 10 days	Not done	Epithelization Softening	Radical with nodes	Epid. Ca., gr 2 Pseudo mucinous cystadenoma—ovary
7 60 W	Asymptomatic Small erosion Early Stage I	Ca. *in situ*	None	2 Courses 18.5 gm. in 74 doses over 103 days	8.4%	Cervix less vascular	Radical with nodes	Chronic cervicitis Endometrial polyp Endometriosis of uterus
8 50 C	Crater Stage I	Epid. Ca., gr 2	Radium 1933 for menorrhagia	6.0 gm. in 28 days	8.5%	Crater filling Epithelization Softening	Radical with nodes	Epid. Ca., gr 2 plus radiation effect

9 62 W	Ulcer Residual from Stage I	1948, Epid. Ca. 1949, Epid. Ca., gr 3	1948 X-ray and radium full course	1.2 gm. in 12 days	Not done	Epithelization Softening	Radical with nodes Cystectomy Bilat. uret transplant	Epid. Ca. vagina Radiation effect Adenomatous polyp of endometrium
10 58 C	Crater Stage IV	Epid. Ca., gr 3	1948 subtotal	3.15 gm. in 21 days	6%	Bleeding ceased Softening Deceased in post op uremia	Stump with nodes Cystectomy Vaginectomy uret. Transplant.	Epid. Ca., gr 3 Cystitis Hydrosalpinx
11 31 W	Sterility Asymptomatic cystic Cx Early Stage I	Sq. Ca., gr 2	None	7.25 gm. in 31 days	Not done	Cervix regained normal appearance	Radical with nodes	Ca. *in situ* Prolif. endometrium hemorrhagic corpus luteum
12 61 C	Stage III	Epid. Ca., gr 2	None	16 gm. in 64 days	13%	Discharge diminished Bleeding ceased Deceased—Post op. Acidosis x abscess	Radical with nodes Cystectomy Bilat. uret. Transplant	Epid. Ca., gr 2 Autopsy: Pelvic abscess
13 65 C	Ulcer Stage III	Epid. Ca., gr 3	None	12.5 gm. in 50 days	5.5%	Discharge and bleeding ceased Epithelization Softening Pulmonary infarcts	Radical with nodes Vena caval ligation	Epid. Ca., gr 2
14 53 C	Crater Stump Stage III	Epid. Ca., gr 2	Subtotal at age 17	7.425 gm. in 61 days	19%	Bleeding ceased Crater healed Pain less; then ceased 6 M Post op. Well	Radical stump plus nodes Cystectomy Vaginectomy Uret. transplant	Epid. Ca., gr 2
15 36 C	Bleeding erosion Stump Stage I	1948 Epithelial Hyperplasia 1950 Ca. *in situ*	Subtotal at age 34	7.25 gm. in 31 days	Not done	Bleeding ceased Lesion cleaner	Radical with nodes	Epid. Ca., gr 1
16 35 C	Vag. disch—2 mo Exophitic Stage II	Epid. Ca., gr 3	None	16.25 gm. in 65 days	Not done	Normal menst. under prog. 2 x Epithelization Softening	Radical plus nodes	Epid. Ca., gr 3
17 53 C	Erosion Stage III	Epid. Ca., gr 3	None	42.5 gm. in 149 doses in 170 days	3.3%	Bleeding stopped Epithelization	None	None

[1] Stage—Indicates League of Nations classification of the disease

regularly before treatment continued to menstruate during the course of such massive dosage with progesterone (table 1).

The percentile recovery of the total dose of administered progesterone in the form of urinary pregnandiol is tabulated for 11 cases in table 1. These figures are in good agreement with comparable data reported by Marrian and Sommerville (*13*) for normal adult females. This indicates that by this admittedly crude measure, the patient suffering from cervical carcinoma metabolizes progesterone in a grossly normal manner.

We do not consider the regressive changes observed to be sufficient to indicate the use of progesterone as a therapeutic agent in carcinoma of the cervix. Nevertheless, these effects do indicate that cervical carcinoma can be substantially altered by steroid hormone administration. In some instances these changes have been somewhat comparable to the hormone-induced regressions seen in breast carcinoma under estrogen therapy (*1*). Such phenomena constitute a chemotherapeutic lead, indicating the general class of compound which may in time prove efficacious as a truly therapeutic agent. In this connection, Lipschutz (*14*) has surveyed a large series of steroids for "anti-tumorigenic action." He concludes that antitumorigenic action is not related to biological hormonal activity. He adds that "there is place for the hope that anti-tumorigenic steroids having no other pharmacological action may be obtained by chemical synthesis."

Our limited observations reviewed in the light of these experimental findings will serve as a basis for further study of the effect of this family of steroid compounds upon the clinical manifestations of cervical cancer.

REFERENCES

(*1*) NATHANSON, I. T.: The relationship of hormones to diseases of the breast. *In* Endocrinology of Neoplastic Diseases. Oxford University Press, New York. p. 138, 1947.

(*2*) HUGGINS, C.: Anti-androgenic treatment of prostatic cancer in man. *In* Approaches to Tumor Chemotherapy. Am. Assn. Adv. Sc., Washington, D. C. p. 379, 1947.

(*3*) OVERHOLSER, M. D., and ALLEN, E.: Atypical growth induced in cervical epithelium of monkey by prolonged injections of ovarian hormone combined with chronic trauma. Surg. Gynec. & Obst. 60: 129–136, 1935.

(*4*) HISAW, F. L., and LENDRUM, F. C.: Squamous metaplasia in the cervical glands of the monkey following oestrin administration. Endocrinology 20: 228–229, 1936.

(*5*) ENGLE, E. T., and SMITH, P. E.: Some uterine effects obtained in female monkeys during continued oestrin administration, with special reference to cervix uteri. Anat. Rec. 61: 471–483, 1935.

(*6*) LIPSCHUTZ, A., MURILLO, R., and VARGAS, L., Jr.: Antitumorigenic action of progesterone. Lancet 2: 420–421, 1939.

(*7*) GOODMAN, A. L.: Progesterone therapy in uterine fibromyoma. J. Clin. Endocrinol. 6: 402–408, 1946.

(*8*) SEGALOFF, A., WEED, J. C., and PARSON, W.: Progesterone therapy of uterine fibromyomas, J. Clin. Endocrinol. 6: 699–700, 1946.

(*9*) GARDNER, W. U., ALLEN, E., SMITH, G. M., and STRONG, L. C.: Carcinoma of the cervix of mice receiving estrogens, J. A. M. A. 110: 1182–1183,, 1938.

(*10*) HERTZ, R., LARSEN, C. D., and TULLNER, W. W.: Inhibition of estrogen-induced tissue growth with progesterone. J. Nat. Cancer Inst. 8: 123–126. 1947.

(*11*) CROSSEN, H. S., and CROSSEN, R. J.: Disease of Women. C. V. Mosby Co., St. Louis, 1935.

(*12*) SOMMERVILLE, I. F., GOUGH, N., and MARRIAN, G. F.: Quantitative determination of small amounts of pregnandiol in human urine. J. Endocrinol. 5: 247–257, 1948.

(*13*) SOMMERVILLE, I. F., MARRIAN, G. F., DUTHIE, J. J. R., and SINCLAIR, R. J. G.: Abnormality in steroid metabolism associated with rheumatoid arthritis. Lancet 258: 116–117, 1950.

(*14*) LIPSCHUTZ, A., IGLESIAS, R., BRUZZONE, S., FUENZILDA, F., and RIESCO, A.: New experimental aspects of the antifibromatogenic action of steroid hormones. Texas Reports on Biology and Medicine 6: 3–20, 1948.

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FIGURE 1.—Case #16. Initial appearance of lesion.

FIGURE 2.—Case #16. After 65 days on progesterone.

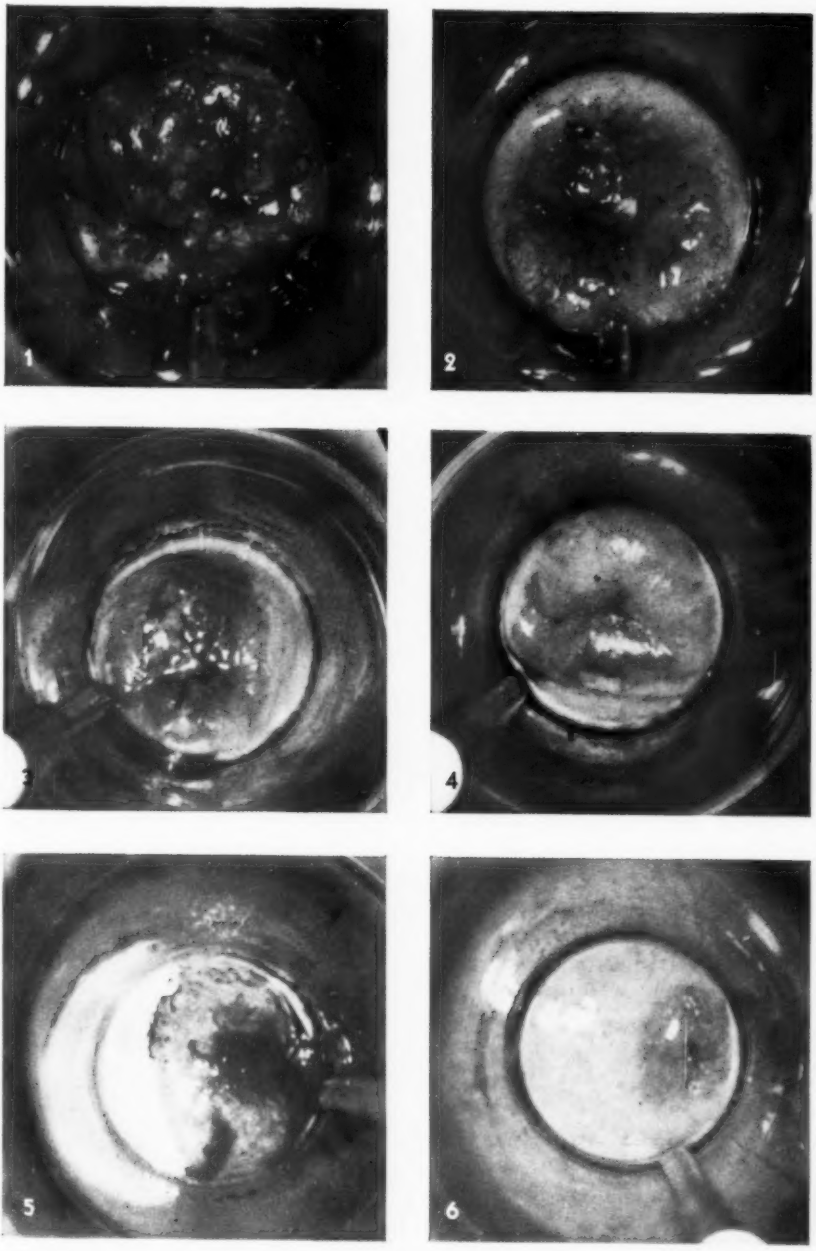
FIGURE 3.—Case #11. Initial appearance of lesion.

FIGURE 4.—Case #11. After 31 days on progesterone.

FIGURE 5.—Case #13. Initial appearance of lesion.

FIGURE 6.—Case #13. After 50 days on progesterone.

*Photographs represent actual size of lesion.



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STUDIES ON THE BLOOD SUPPLY OF TUMORS IN MAN. I. FLUORESCENCE OF CUTANEOUS LESIONS [1] [2]

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The continued growth of most neoplasms is dependent in part upon the blood supply. The vascular reaction about neoplastic transplants in mice appears shortly after the implantation (*1, 2*). Wood *et al.* (*3, 4*) demonstrated an increased blood supply *via* the bronchial arteries in primary malignancies of the lung in contradistinction to metastatic pulmonary tumors, which receive their blood mainly from the pulmonary arteries. The vascularity of some tumors is a well known fact and, at operation, excessive enlargement of arteries leading to tumor areas is frequently noted. Moore (*5*) has demonstrated an apparent affinity of brain tumors for fluorescein, *in vivo*, and recently, Breedis and Young (*6*) have shown by perfusion of the liver obtained at post mortem an apparent preferential arterial blood supply of spontaneous, transplanted, or metastatic neoplasms in this organ, in contrast to the predominantly venous supply to the uninvolved parenchyma. These findings have been confirmed in this laboratory.[5] This report deals with studies *in vivo* of the afferent blood supply of neoplasms and other lesions in the skin as demonstrated by fluorescein.

METHOD

Eighteen patients with neoplastic lesions involving, or presenting through, the skin were studied. Three to ten cubic centimeters of 5 percent fluorescein [6] were injected rapidly into a major artery leading directly toward the tumor site. In 3 patients the fluorescein was given intravenously on 5 occasions. The time of appearance and duration of the fluo-

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[4] American Cancer Society Fellow.

[5] Ortega, P., and Bierman, H. R.: Unpublished data.

[6] Fluorescein, 5 percent, with sodium bicarbonate, 5 percent (Fluorescite). C. F. Kirk Company, New York, N. Y.

rescence was observed under ultraviolet light. The areas of fluorescence were outlined with indelible ink on the skin under ultraviolet light and, after the room was relighted, the relationship to the active lesions was recorded. In some instances, the fluorescein was injected through an intra-arterial catheter (*7*). In most cases, however, the dye was injected directly into a large peripheral artery, *e. g.*, femoral or brachial.

RESULTS

Eighteen patients were studied with 37 intra-arterial and 5 intravenous injections of 5 percent fluorescein (table 1). In general, the neoplastic lesions directly involving skin (mycosis fungoides, lymphoepithelioma) began to fluoresce a bright yellow color within 3 to 15 seconds after introduction of the fluorescein into the artery. This color further intensified during the injection period, and after 2 to 4 minutes, the normal tissue adjoining the lesions exhibited no fluorescence or only a minimal amount. (figs. 1 and 2). The luminescence of the neoplastic lesions lasted for 1 or 2 hours, with slight decrease in intensity during this period. The fluorescence of histamine wheals employed as controls disappeared within 20 minutes, as did most of the fluorescence in the uninvolved skin. In 4 cases where cutaneous ulceration existed, fluorescent material was wiped from the open lesions shortly after intra-arterial injection. Fluorescence of areas and lesions in other parts of the body of these patients was barely discernible. In 2 cases (GH, HA) new metastatic lesions showed moderate uptake of the dye after recirculation. Old necrotic lesions exhibited no luminescence (figs. 3 and 4). Lesions that had been previously treated intensively by X-ray or nitrogen mustard fluoresced poorly or not at all (figs. 5 and 6). The lesions of Kaposi's sarcoma showed no fluorescence (figs. 7 and 8).

Fluorescein in identical amounts was injected intravenously before, and twice after, 15 mg. of triethylmelamine was administered orally in one case (HA), on three separate occasions. Initially, the cutaneous lesions showed fluorescence after 90 seconds with the intensity reaching its maximum 2 minutes thereafter and persisted for 2 hours. There was a marked decrease in luminescence of the same lesions following the second injection of fluorescein as compared with the initial injection before melamine was administered 5 days previously. The third injection of fluorescein, 29 days later, showed a return to the original fluorescence.

In general, the fluorescence was limited to the neoplastic lesions, closely following their borders (table 1). In a few instances, however, there was a discrepancy between the fluorescene and the visible limits of the lesions of mycosis fungoides in that the brilliance of the yellow color varied within the lesions and occasionally was out of the area of a visible lesion. There was no uptake of intravenously administered fluorescein by lesions that were thought to be infiltrations of Hodgkin's disease. It was also possible to prevent fluorescence of an area completely, either involved or normal, by manual pressure, presumably by obliterating the capillaries.

In four instances there was an absence of fluorescence in areas previously treated with intra-arterial nitrogen mustard.

The disappearance of luminescence after 2 hours was gradual and in proportion to the intensity; *i. e.*, it took longer for those lesions that fluoresced brightly to lose the dye than those with less brilliance. The decrease in luminescence occurred uniformly throughout the lesion, although in a few instances the center of the lesions lost the yellow color first and the periphery lost it last.

DISCUSSION

Algire (*1*) has found that proliferation of vascular tissue develops promptly about a neoplasm during its enlargement. Similarly, the arterial blood supply to some neoplasms is greater, in proportion, than to comparable normal tissues of the same volume (*8*). One must interpret the findings first, from the time of initial fluorescence and, second, from its later intensification. Since the material was introduced into the largest artery leading into the involved leg, and since in most instances the appearance of the fluorescence in and about the neoplastic lesion appeared within 3 to 15 seconds after the injection was initiated and many seconds before it was seen in the uninvolved areas (depending upon the rate of injection), it is most suggestive that the afferent blood supply to those neoplastic lesions is increased. In addition, the further intensification of the fluorescein in a neoplastic lesion under direct observation, is probably due to an altered vascular permeability. It is, therefore, reasonable to anticipate that either radiopaque media or fluorescein would reach the neoplastic tissue by arterial administration in greater concentration than uninvolved areas. This has already been shown to be true in the vascular patterns demonstrated by arteriography of intracranial and visceral neoplasms (*7*, *9*). The rapid and apparently selective appearance of relatively large amounts of fluorescein in the lesions of mycosis fungoides following arterial injection is likewise indicative of an increased arterial blood supply to the involved areas. Such vascularity is not limited exclusively to tumors nor does it occur in all neoplasms as was demonstrated in these studies. The prompt appearance of fluorescein in the lesions supplied by the artery into which the dye had been given is not unusual or unexpected. However, the marked luminescence evident in and sharply confined to the lesion as compared to the surrounding uninvolved skin or histamine wheal is indicative of a characteristic, increased, arterial blood supply.

The persistence of the fluorescence in the lesions long after it had vanished in the histamine wheals and uninvolved skin, is suggestive of increased capillary permeability within the immediate vascular supply of the lesion. It would appear reasonable to expect an increased diffusion rate and altered capillary permeability to operate mostly during the slower phase of increasing intensity, and when any material injected into an artery leading to any tissue appears in greater concentration in the tumor (as judged under direct vision) before it does in the immediate

TABLE 1.—*Injection of 5 percent fluorescein in patients with visible neoplastic cutaneous lesions*

Patient	Age	Sex	Diagnosis	Injection Site	Remarks
A. P.	58	M	Mycosis fungoides. Previously treated with X-ray and nitrogen mustard.	Right femoral artery	Prompt fluorescence of new lesions. No fluorescence of treated lesions. No fluorescence by recirculation.
				Right femoral artery	
				Left femoral artery	
				Right brachial artery	
				Right femoral artery	
				Right aorta at A4	
F. S.	38	M	Mycosis fungoides	Right femoral artery	Fluorescence in active lesions. None in previously treated lesions. Minimal fluorescence after recirculation.
				Left femoral artery	
				Left femoral artery	
				Right and left femoral artery	
				Right femoral artery	
				Right femoral artery	
A. C.	51	F	Mycosis fungoides	Right femoral artery	Marked fluorescence of active lesions, moderate fluorescence of inactive areas, in X-ray treated areas. No fluorescence after recirculation.
				Left femoral artery	
				Right femoral artery	
				Left femoral artery	
G. H.	38	F	Melanosarcoma, metastatic to left leg.	Left femoral artery	Fluorescence of new lesions that were enlarging rapidly. None in soft necrotic areas of lesions.
J. V.	38	M	Mixed tumor of the parotid, right, with involvement of right half of tongue.	Right common carotid	Prompt fluorescence of lower half of neoplastic lesion, and right half of tongue.
T. F.	8	F	Hemangioendothelial sarcoma. Intensive X-ray therapy.	Left vertebral and thyrocervical axis.	Fair fluorescence of general area with more deep fluorescence observed in untreated tumor areas.
				Left external carotid	No fluorescence in heavily irradiated areas.
				Left internal carotid	Fluorescent material obtained from ulcer.
M. D.	46	M	Basal cell carcinoma of face, right, treated intensively with X-ray and surgical excision.	Right external carotid by catheter.	No fluorescence in treated area. Light fluorescence about the advancing edge of the neoplasm deep in the open area.
G. Z.	9	M	Lymphoepithelioma, right tonsillar fossa—prior to intensive X-ray therapy.	Right external carotid by catheter.	Fluorescence limited to intra-oral tumor. Right supratonsillar area.

H. A.	71	M	Epidermoid carcinoma of left antrum with cutaneous metastases.	Right cubital vein	Marked fluorescence limited to erythematous cutaneous lesions within 90 seconds.
				Left cubital vein	5 days after melamine therapy, fluorescence of cutaneous lesions markedly less than originally.
				Right cubital vein	29 days later fluorescence approaching original brilliance.
D. G.	23	F	Hodgkin's granuloma	Right femoral vein	No fluorescence of lesions on leg.
M. S.	66	M	Kaposi's sarcoma	Right femoral artery	No fluorescence of cutaneous lesions.
				Right foot vein	No fluorescence of cutaneous lesions.
G. M.	40	F	Epidermoid carcinoma, nose, pharynx, right—X-ray treatment, intensive.	Right common carotid by catheter.	No increased uptake of P^{32} or fluorescein.
				Right common carotid by cut-down.	No increased uptake of P^{32} or fluorescein.
T. M.	44	M	Carcinoma of the lung	Hepatic artery by catheter	No fluorescence of subcutaneous lesions.
F. G.	62	M	Epidermoid carcinoma of the lip, metastatic. Treated intensively with X-ray.	Left superficial femoral artery	No lesions directly involving skin. Treated previously with guanazolo, and developed an erythematous eruption on extremities. Prompt fluorescence throughout eruption. Marked in area of intra-arterial injection. Three minutes later fluorescence started peripherally in lesions obtaining dye after recirculation. No fluorescence of neoplastic area of left mandible and neck.
J. S.	60	M	Epidermoid carcinoma of nasopharynx, metastatic to right cervical region. Treated previously with X-ray.	Right external carotid by cut-down.	Moderate fluorescence of ulcerated tumor nodule plus outline of circulation of external carotid artery.
S. E.	66	M	Carcinoma of bladder, metastatic to skin, with lymphangitic spread to subcutaneous tissues of torso, pelvis, and thighs.	Aorta at level of T_4 by catheter.	Prompt fluorescence of cutaneous chest lesions, followed by fluorescence of inguinal lesions. Many subcutaneous and cutaneous infiltrative lesions did not fluoresce.
G. B.	48	M	Adenocarcinoma of left kidney	Aorta at level of L_1 by catheter	Minimal fluorescence of skin areas supplied.
				Aorta at level of T_4 by catheter	Minimal fluorescence of skin areas supplied.
E. U.	31	M	Carcinoma of the testes	Right internal mammary. Right thyrocervical axis.	Prompt fluorescence of skin areas midline sternal supplied. None elsewhere for 10–15 minutes. Subcutaneous metastasis fluoresced only on right half.

involved area, within a matter of a few seconds, it would appear that it does so because there are larger, and more, channels leading to that tumor.

In 4 instances where the tumor nodules were ulcerated, fluorescent material was easily demonstrated on cotton swabs that had been gently drawn across the surface of the ulcer. Whether this apparent increased permeability of the vessels within the tumor is responsible for the alleged preference of neoplasms for fluorescein (*5*) or whether the tumor itself exerts some selective influence upon substances circulating through tumor tissue, cannot be proved at present. Some neoplasms show a selective uptake of radiophosphorus (P^{32}), which persists for 24 to 48 hours (*10*, *11*). Inflammatory lesions of the breast show an even greater differential uptake for radiophosphorus. The increased vascularity and altered capillary permeability of such lesions must be considered in an interpretation of the selectivity of neoplasms for injected materials.

While it is difficult to state quantitatively the intensity of fluorescence, it is nevertheless possible to determine the relative difference of brightness in comparable areas. The intensity of the fluorescence under these conditions appears to be directly related to the amount and concentration of dye that is injected. It is also apparent that lesions supplied arterially with fluorescein in high concentration exhibited luminescence throughout, while lesions beyond the direct arterial blood supply received the fluorescein in much lower concentrations after recirculation, and fluoresced proportionately less and then only at the periphery. After 30 minutes to 1 hour, these lesions showed luminescence throughout but always much less in degree than those lesions receiving the dye by direct arterial injection.

The absence of fluorescence of lesions previously treated with X-ray irradiation, confirms the fact the X-ray irradiation decreases the vascular supply of neoplasms, reducing its main nutritional source and consequently curtailing its rate of enlargement or reducing its mass until the necessary level of vascular supply is re-established. Lesions previously treated with intra-arterial nitrogen mustard similarly failed to fluoresce.

Although it has been confirmed that arteries leading to neoplastic areas are frequently enlarged and increased in number, the fundamental cause for this proliferation remains obscure. That the vascular proliferation results in an increase of oxygenated blood to neoplasms, which can metabolize under relatively anaerobic conditions, appears to be unnecessary. However, the rate of transfer and utilization of this increased oxygen supply remains obscure.

The increased vascular reaction about neoplasms appears to be at the advancing edge of the lesion, or in its most active area. Tumors that enlarge rapidly often push back the increased number of capillaries at the periphery so that a pseudocapsule may be formed about the tumor. Central necrotic areas of tumor nodules show little, if any, fluorescence, which may prove its avascularity and may be responsible for the tissue degeneration. However, some smaller lesions did fluoresce and although they were not as soft as the larger lesions they were probably necrotic.

Figure 4 was taken 20 minutes after the injection of fluorescein. However, if certain necrotic lesions possess connections with the vascularity in the periphery, then it is possible that the fluorescein would make its way into the necrotic area, probably more so in the smaller lesions. This occurred in some ulcerated lesions as noted. It is interesting to note that at the time figure 4 was taken the histamine-wheal control area in the lower part of the leg no longer showed fluorescein. The persistence of fluorescence is probably a function of capillary permeability, including diffusion rate, tissue fixation, *etc.*

Cutaneous neoplastic lesions have been observed to be definitely warmer than the surrounding normal tissue, affording additional evidence of their increased blood flow. The amount of arteriovenous shunting through such a single lesion is of relatively little significance with regard to the entire body; however, with many large, metastatic lesions scattered throughout the individual, particularly in bone, the total amount of arteriovenous shunting may become considerable. These shunts and their effect on the cardiovascular dynamics of the patient have already been demonstrated in extremities (*12*). Such vascularity is not limited exclusively to tumors.

If such increased arterial proliferation is present, it may be advisable to administer the most promising chemotherapeutic agents by arterial routes leading to the neoplasm (*13*). Higher concentrations may be attained in the blood leading to the involved area by this procedure. Whether any of the chemotherapeutic agents will affect the tumor proportionately to their concentration remains to be determined. Furthermore, the length of time the therapeutic agent is kept in contact with the lesion is obviously of considerable importance. Substances that are removed promptly in the lesions, or that expend their effect rapidly (*e. g.*, nitrogen mustard and short-lived radioisotopes), or that directly affect the capillary bed of the tumor [such as bacterial polysaccharides (*14*)] offer desirable approaches. Methods to slow the venous return or enhance the afferent flow may further exploit this suggestion for therapy.

The data obtained demonstrate the necessity of determining the presence and amount of afferent supply to the lesions to be treated. Previous therapy by irradiation, nitrogen mustard, triethylmelamine, and bacterial polysaccharides, at least in some instances, may reduce the arterial supply to cutaneous neoplasms. It is also likely that some cutaneous neoplasms will not possess an increased arterial blood supply. If intra-arterial therapy is contemplated, some measure of the vascularity of the lesion should be determined either by dye, radioisotope, temperature, or other suitable means.

SUMMARY

Eighteen patients with cutaneous lesions, mostly neoplastic, have been studied following 42 intravascular injections of fluorescein.

The arterial blood supply to most primary, cutaneous tumor areas was demonstrated to be greater than to normal tissues as judged by the

fluorescein appearing in tumor areas more quickly, remaining longer, and in greater concentration than in surrounding tissues. Necrotic metastatic lesions did not fluoresce as promptly nor as brightly as those of mycosis fungoides or lymphoepithelioma.

Previous surgery, X-ray irradiation, nitrogen mustard, and, in one case, therapy with triethylmelamine materially decreased the fluorescence of cutaneous lesions.

The suggestion was made that some chemotherapeutic agents may be more advantageously given *via* the arterial route, delivering higher concentrations directly to appropriate neoplastic tissues. It is essential that the vascularity to lesions be determined before intra-arterial administration of these chemotherapeutic agents is attempted.

REFERENCES

(*1*) ALGIRE, G. H., and CHALKLEY, H. W.: Vascular reactions of normal and malignant tissues *in vivo*. I. Vascular reaction of mice to wounds and to normal and neoplastic transplants. J. Nat. Cancer Inst. 6: 73–85, 1945.

(*2*) SPITZ, K.: Über Blutungsreaktionen am Impfcarcinom der Maus. Ztschr. f. Krebsforsch. 40: 50–70, 1933.

(*3*) WOOD, D. A., CREVER, J. W., and MILLER, M.: Changes in the dual circulation of human lungs in various pathological conditions. J. Tech. Methods 17: 78–90, 1937.

(*4*) WOOD, D. A., and MILLER, M.: The role of the dual pulmonary circulation in various pathologic conditions of the lungs. J. Thoracic Surg. 7: 649–670, 1938.

(*5*) MOORE, G. E.: Fluorescein as an agent in the differentiation of normal and malignant tissues. Science 106: 130–131, 1947.

(*6*) BREEDIS, C., and YOUNG, G.: Blood supply of neoplasms in the liver. Am. Soc. Exper. Path. Fed. Proc. 8: 351, 1949.

(*7*) BIERMAN, H. R., MILLER, E. R., BYRON, R. L., Jr., DOD, K., BLACK, D., and KELLY, K.: Intra-arterial catheterization in man. Bull. Univ. Calif. Med. Center 1: 84–88, 1949.

(*8*) WILLIS, R. A.: Pathology of Tumours. C. V. Mosby Company, St. Louis, p. 134, 1948.

(*9*) HODES, P. J., PERRYMAN, C. R., and CHAMBERLAIN, R. H.: Cerebral angiography. Am. J. Roentgenol. 58: 543–583, 1947.

(*10*) MCCORKLE, H. J., LOW-BEER, B. V. A., BELL, H. G., and STONE, R. S.: Clinical and laboratory studies on the uptake of radioactive phosphorus by lesions of the breast. Surgery 24: 409–415, 1948.

(*11*) STEINBERG, D., and SELVERSTONE, B.: Radioautography of cerebral tumors employing P^{32}. Proc. Soc. Exper. Biol. & Med. 74: 304–308, 1950.

(*12*) BIERMAN, H. R., and KELLY, K.: Arteriovenous shunts in metastatic neoplasms of man. In preparation.

(*13*) BIERMAN, H. R., BYRON, R. L., Jr., MILLER, E. R., and SHIMKIN, M. B.: Effects of intra-arterial administration of nitrogen mustard. Abstract. Am. J. Med. 8: 535, 1950.

(*14*) ALGIRE, G. H., LEGALLAIS, F. Y., and PARK, H. D.: Vascular reactions of normal and malignant tissues *in vivo*. II. The vascular reactions of normal and neoplastic tissues of mice to a bacterial polysaccharide from Serratia marcescens (Bacillus prodigiosus) culture filtrates. J. Nat. Cancer Inst. 8: 53–62, 1947.

PLATES

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PLATE 92

FIGURE 1.—F. S. Male, age 38. Mycosis fungoides. Extensive lesions involving both lower extremities. Lesions of right upper thigh were previously treated by intra-arterial nitrogen mustard.

FIGURE 2.—F. S. Under ultraviolet light fluorescein injected in the right femoral artery. Note intensive luminescence of the active lesions of midthigh and below the knee and the absence of fluorescence in the upper right thigh in the area previously treated with arterial nitrogen mustard.

FIGURE 3.—G. H. Female, age 38. Melanosarcoma. Extensive skin metastases.

FIGURE 4.—G. H. Under ultraviolet light lateral area of left thigh following the injection of fluorescein into the left femoral artery. Intensive fluorescence in the new lesions with absence of fluorescence in the large necrotic lesions within the luminescent area of the thigh and leg.

FIGURE 5.—A. C. Female, age 51. Mycosis fungoides. Intensively treated with X-ray therapy and intravenous nitrogen mustard.

FIGURE 6.—A. C. Under ultraviolet light following administration of fluorescein into the right femoral artery. Intensive fluorescence of new lesions without fluorescence in most of the area of the extremities. This film was overexposed to accentuate the minimal fluorescence of the skin.

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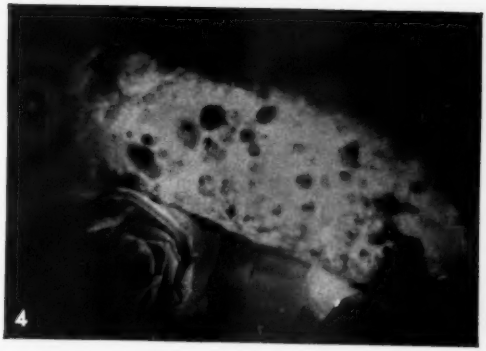
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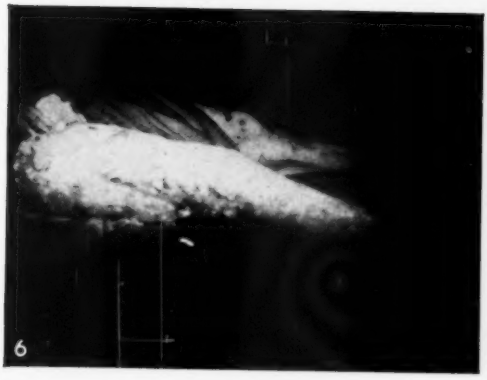
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PLATE 93

FIGURE 7.—M. S. Male, age 66. Kaposi's sarcoma.

FIGURE 8.—M. S. Absence of fluorescence of lesions of Kaposi's sarcoma following arterial injection of fluorescein.

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STUDIES ON THE BLOOD SUPPLY OF TUMORS IN MAN. II. INTRA-ARTERIAL NITROGEN MUSTARD THERAPY OF CUTANEOUS LESIONS[1] [2]

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The arterial blood supply of some cutaneous neoplasms has been shown to be increased, affording a direct route for the administration of chemotherapeutic drugs (*1*, *2*, *3*). In addition, the capillaries within some neoplasms possess permeability that allows the prompt and prolonged appearance of fluorescein and certain radioisotopes in the lesions (*1*, *4*, *5*). Such enhanced passage of intra-arterial substances into tumor tissue may also pertain to chemotherapeutic materials. The administration of substances into the proper artery would direct a high concentration of these materials to those lesions that have an increased arterial vascularity. The preference or selectivity of various agents for specific tumors may further enhance the concentration of such an agent in the tumor if, in addition, the agent is presented directly to the lesion (*4*). The toxic effects of chemotherapeutic substances upon the rest of the body (*e. g.*, bone marrow, liver, kidneys, *etc.*) consequently may be materially reduced, since an appreciable amount of the agent may be fixed in the tumor tissue and the remainder would be well diluted during recirculation. The amine mustards meet these criteria and were selected for the initial studies.

Methyl-bis (beta-chloroethyl) amine (HN2)[5] has been given intravenously in many clinics over the past 5 years and the hematologic and therapeutic effects have been well described (*6*). This report deals with the therapeutic and hematologic changes observed following the intra-arterial administration of methyl-bis (beta-chloroethyl) amine (HN2) in patients with primary or metastatic neoplastic lesions involving the skin.

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[2] Supported in part by research grant C-396 from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service, and the American Cancer Society, Inc., Institutional Grant 43.

[3] Damon Runyon Fellow.

[4] American Cancer Society Fellow.

[5] Hereinafter also referred to as nitrogen mustard and HN2. Kindly furnished by Merck & Company, Rahway, N. J.

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METHODS AND PATIENTS

Seventeen patients who had cutaneous lesions that could be visually observed and measured were selected for this study. The clinical and hematologic status of the patients was carefully documented prior to the administration of HN2 and followed closely thereafter. The appropriate brachial or femoral artery was injected when lesions of the extremities were available for study. Elsewhere, intra-arterial catheters were manipulated into the appropriate artery (*7*). Fluorescein was injected first to determine the vascularity of the lesions whenever feasible (*1*).

The amine mustard (HN2) was dissolved in normal saline and administered promptly in a concentration of 1 mg. per cc. at a rate of approximately 10 mg. per minute.

RESULTS

A total of 42 separate injections of HN2 (5 mg. to 80 mg., 0.1 to 1.0 mg. per kg.) were given to 17 hopelessly incurable patients with far advanced neoplastic lesions involving the skin. Twenty-seven infusions were given intra-arterially and 15 intravenously; 6 patients had both intravenous and intra-arterial injections.

There was no subjective complaint of discomfort during the administration of the nitrogen mustard into arteries in any instance, and no evidence of irritation at the injection site in arteries was detected at post-mortem examination in 10 patients. Three patients are living, and permission for examination in the remaining 4 patients could not be obtained. In general, improvement was most marked in those lesions that previously had fluoresced brightly. Those lesions previously treated intensively by X-ray or intra-arterial HN2 did not reveal an increased vascularity, and correspondingly showed little response to the intra-arterial HN2.

The effects of the intra-arterial administration of nitrogen mustard in mycosis fungoides was prompt and dramatic in those areas injected in three of four patients (figs. 1 and 2). The raised erythematous lesions flattened within 4 to 7 days, became indurated, and faded to a dull brown color in the subsequent 2 weeks. This was in contrast to the intravenously administered nitrogen mustard in which the effects on the lesions were more gradual, less marked, and of shorter duration. In the extremities treated with HN2 intra-arterially, no new lesions appeared in previously involved areas but recurrences were observed later in previously unaffected areas. The new lesions did not encroach upon the areas previously resolved by the intra-arterial nitrogen mustard. The resolved lesions also failed to fluoresce following intra-arterial administration of 10 cc. of 5 percent fluorescein. The fourth patient (AC), who exhibited little improvement after intra-arterial HN2, had previously been treated intensively with X-ray and intravenous HN2 and showed increased vascularity with fluorescein in only a few lesions. These vascular lesions promptly improved following intra-arterial HN2.

Nitrogen mustard, 0.3 to 0.4 mg. per kg., administered to the lower extremities *via* the femoral artery produced no swelling or hyperesthesia of the extremity distal to the site of the injection. Swelling of the tissues of the forearm appeared distal to the site of brachial-artery injection in one instance (FA) within 8 hours after the administration of 0.3 mg. of nitrogen mustard per kg. of body weight. There was an associated hyperesthesia persisting for 2 or 3 days, but no changes occurred in the radial pulse. All swelling subsided within 10 days, and improvement in the lesions of the forearm became quite evident, which was in contrast to the result obtained in this patient following the intravenous injection of the same dose (0.3 mg. per kg.) of nitrogen mustard.

In four patients who had open ulcerated areas, fluorescent material injected intra-arterially prior to the administration of the therapeutic agent could be wiped from the ulcerated surfaces. These also were found to respond favorably to HN2 administered into the same artery. One patient (AP) had such an area of approximately 200 sq. cm. on the lower extremity (fig. 3). Injection of HN2, 0.3 mg. per kg. of body weight, into the left femoral artery was followed by prompt improvement and epithelization (fig. 4).

In one individual (EU) a catheter was introduced into the right internal mammary artery from the left femoral artery (7). Diodrast (70 percent) injected through the catheter revealed the blood supply to the tumor mass overlying the sternum (fig. 5). The skin supplied by this artery, including the right half of the metastatic mass over the sternum, was outlined following the injection of 5 percent fluorescein through the catheter (fig. 6). Nitrogen mustard, 0.6 mg. per kg. of body weight, was then injected into this artery. The catheter was next moved to the right thyrocervical axis, where injections of Diodrast (35 percent) and fluorescein demonstrated the areas supplied by this artery, including a metastatic tumor in the right trapezius muscle (fig. 7). Nitrogen mustard, 0.4 mg. per kg. of body weight, was injected into the right thyrocervical artery. During the next few hours erythema and diaphoresis appeared in the skin areas that had fluoresced. Bullae appeared the next day (fig. 8), and the areas progressed rapidly to a slough resembling that from a third degree burn (figs. 9 and 10). The tumor mass of the right-shoulder area completely disappeared within 6 days, and the sternal mass decreased in size from 6 by 4 cm. before treatment to 2 by 1 cm. within a week following therapy (fig. 9). Repeated biopsies from both right and left halves of the tumor revealed only necrotic material in the remaining mass. The mass in the trapezius muscle did not reappear, but the sternal tumor mass recurred in approximately 1 month (fig. 10).

One other slough was observed in a 36-year-old woman with a frozen pelvis from an advanced carcinoma of the cervix who received HN2 *via* an intra-arterial catheter. Previous surgery had shunted much of the blood flow away from the pelvis to the skin areas. Fluorescein brilliantly outlined the surface area of supply by the inferior gluteal artery. A large part of the 50 mg. of HN2 administered reached this skin area and after

an immediate period of erythema and vesiculation a large nonhealing slough resulted within 3 weeks.

In all cases, routine blood counts were done at frequent intervals. The total leukocyte counts were somewhat variable, but in the five patients treated with alternate intravenous and intra-arterial HN2, the depression of the leukocyte count following the intra-arterial HN2 was markedly less than that observed after the intravenous injections. In addition, the duration of the leukopenia was shorter following the intra-arterial administration than by the intravenous route.

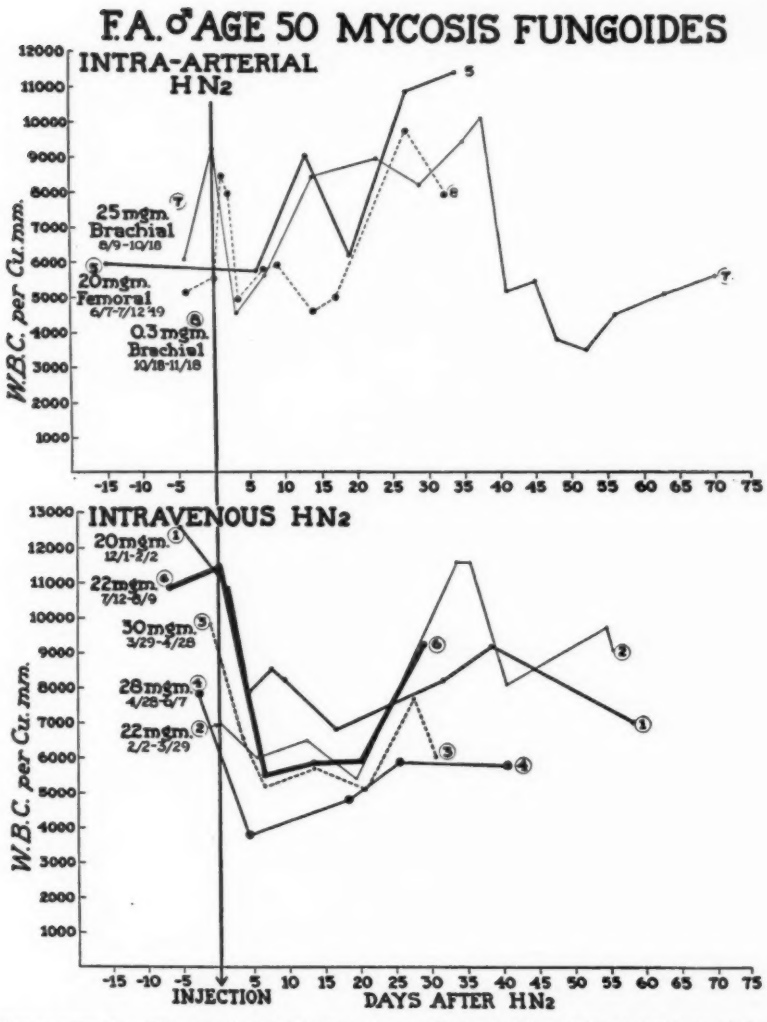
In one patient (FA) who received four consecutive intravenous injections of nitrogen mustard, the third and fourth injections produced marked and prolonged depression of the number of leukocytes. The fifth injection of nitrogen mustard was given intra-arterially and resulted in no leukopenia. A sixth injection was given intravenously, resulting in a moderate depression of the leukocyte count for a long period. A seventh and last injection was intra-arterial and produced little effect on the leukocyte count (text-fig. 1).

Another patient (AP) received 0.3 mg. of nitrogen mustard per kilogram of body weight into the right femoral artery with only a slight depression of the total white count. Fourteen days later he was given the same dose intravenously, which resulted in a marked and prolonged depression of the total white-cell count. A repeat injection in 25 days into another artery resulted in a transient, small decrease in total white-cell count. In order to compare the effect of successive intra-arterial doses, another arterial injection of HN2 was made 24 days later. This resulted in an increase in the number of circulating leukocytes. Thirteen days later another intravenous injection of HN2, 0.3 mg. per kg., was given, resulting in a marked decreased in the total leukocyte count. This patient was then given the same dosage of HN2 intra-arterially 17 days following the previous intravenous administration. This resulted in a depression of the white-cell count, which returned to original level in 10 days. Twenty-three days later, the last administration of nitrogen mustard was given into the descending aorta, to determine the effect on the generalized body lesions and to observe variations in the leukocyte count. Improvement of the trunk lesions was not as marked as in the extremities, but was much more than occurred with intravenous administration. The total white-cell count was not depressed, and 10 days later it was above the original level.

DISCUSSION

The increased vascularity of tumors affords exposure of a large volume of tumor to relatively high concentrations of nitrogen mustard when administered directly into the arteries supplying the tumor. This was considered the most important factor for the more marked clinical improvement observed following intra-arterial rather than intravenous nitrogen mustard.

The intra-arterial administration of HN2 in most instances was fol-



TEXT-FIGURE 1.—Changes in total leukocyte counts in peripheral blood following intravenous and intra-arterial HN2. Note initial leukocytosis following intra-arterial administration.

lowed by a shorter and less severe leukopenia than with intravenous therapy. The leukopenia occurred at approximately the same interval after arterial injection as that observed following intravenous nitrogen mustard therapy.

The shift of the hemogram both in the peripheral blood and in the bone marrow was less with intra-arterial than that observed with intravenous administration of HN2. No marked alteration in the bleeding or coagulation time was observed.

Nitrogen mustard is a vesicant when applied to the skin and will thrombose veins if injected in high concentrations. In our studies, no subjective reaction was noted at the time of intra-arterial injection. Postmortem examination of the sites of injection in several cases failed to reveal any evidence of irritation or damage when the arteries were examined grossly and microscopically within periods of a few days to months after injection. The delivery of large amounts of HN2 directly to the skin resulted in tissue destruction resembling third degree burns from heat or X-ray irradiation. This can be obviated by administration of smaller total amounts in less concentration when small local areas of the skin receive the HN2. External pressure on the skin during the injection will also reduce the blood flow through this area and thus prevent nitrogen mustard from reaching the skin.

It was initially anticipated that HN2 would appear free in the ulcerated areas, as did the fluorescein. The result was unexpected for the vesicant action of HN2 was not observed and healing rather than further destruction occurred. The usual nausea and vomiting following nitrogen mustard was observed to be less frequent and of shorter duration following the intra-arterial than after intravenous administration of comparable doses.

The pulmonary circulation is exposed to higher concentrations of substances with intravenous injections than any other capillary bed. Damage to those mechanisms that play an important role in the hematologic system (*8*, *9*) may disturb the homeostatic balance that normally exists and result in the more severe depression of the leukocyte and platelet levels noted with intravenous HN2 therapy. Relatively large doses of HN2 may be administered intra-arterially to a tumor mass avoiding the dilution of the HN2, and by-passing the pulmonary circulation at least for the first circulation. Nitrogen mustard decomposes rapidly, once hydrolyzed, so that recirculation of HN2 following intra-arterial infusion would expose the bone marrow, spleen, and other hematopoietic tissues to a much lower concentration of HN2 than with intravenous therapy.

The administration of nitrogen mustard or other agents should be preceded by a demonstration of the vascularity of the area to be treated. Previous surgery or X-ray therapy of such a degree as to impair the vascularity of the region to be treated is a distinct contraindication to such therapy, particularly if the blood flow is shunted to other more vulnerable areas which may slough.

SUMMARY

Twenty-seven intra-arterial and 15 intravenous injections of methyl-bis (beta-chloroethyl) amine (HN2) were given to 17 hopelessly incurable patients with far-advanced neoplastic lesions involving the skin.

Prompt and marked improvement followed intra-arterial HN2 in all of four patients with mycosis fungoides, in those lesions that possessed increased vascularity. Areas of ulceration in two of these patients healed within 14 days after intra-arterial HN2.

In 19 cutaneous areas involved by various metastatic neoplastic lesions, which exhibited increased vascularity, all showed a prompt decrease in size following intra-arterial HN2. Six areas that failed to exhibit an increased vascularity did not respond to the intra-arterial infusion of HN2.

The intra-arterial injection of HN2 is followed by less severe toxic hematologic changes than after intravenous administration. Those neoplastic lesions reached directly by the intra-arterial HN2 responded more promptly and completely than after intravenous HN2 in those cases in which a valid comparison could be made.

REFERENCES

(1) BIERMAN, H. R., KELLY, K. H., DOD, K. S., and BYRON, R. L., Jr.: Studies on the blood supply of tumors in man. I. Fluorescence of cutaneous lesions. J. Nat. Cancer Inst. 11: 877–890, 1951.

(2) BIERMAN, H. R., BYRON, R. L., Jr., MILLER, E. R., and SHIMKIN, M. B.: Effects of of intra-arterial administration of nitrogen mustard. Abstract. Am. J. Med. 8: 535, 1950.

(3) KLOPP, C. T., BATEMAN, J., BERRY, N., ALFORD, C., and WINSHIP, T.: Fractionated regional cancer chemotherapy. Abstract. Cancer Research 10: 229, 1950.

(4) MCCORKLE, H. J., LOW-BEER, B. V. A., BELL, H. G., and STONE, R. S.: Clinical and laboratory studies on the uptake of radioactive phosphorus by lesions of the breast. Surgery 24: 409–415, 1948.

(5) STEINBERG, D., and SELVERSTONE, B.: Radioautography of cerebral tumors employing P^{32}. Proc. Soc. Exper. Biol. & Med. 74: 304–308, 1950.

(6) SPURR, C. L., SMITH, T. R., BLOCK, M., and JACOBSON, L. O.: The role of nitrogen mustard therapy in the treatment of lymphomas and leukemias. Am. J. Med. 8: 710–723, 1950.

(7) BIERMAN, H. R., MILLER, E. R., BYRON, R. L., Jr., DOD, K., BLACK, D., and KELLY, K.: Intra-arterial catheterization in man. Bull. Univ. Calif. Med. Center 1: 84–88, 1949.

(8) BIERMAN, H. R., BYRON, R. L., Jr., and LANMAN, J. T.: Cross-circulation studies in man. Bull. Univ. Calif. Med Center 1: 89–96, 1949.

(9) HOWELL, W. H., and DONAHUE, D. D.: The production of blood platelets in the lungs. J. Exper. Med. 65: 177–204, 1937.

PLATE 94

FIGURE 1.—F. A., male, age 50. Mycosis fungoides of 7 years' duration. Raised ulcerated, exfoliating lesions of the dorsum of both hands.

FIGURE 2.—F. S., 14 days after 20 mg. of HN2 injected into left brachial artery. Marked healing and disappearance of lesions.

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PLATE 95

FIGURE 3.—A. P., male, age 61. Mycosis fungoides of 12 years' duration. Widely disseminated lesions with ulceration of left leg and lower thigh.

FIGURE 4.—A. P., 16 days after 18 mg. of HN2 injected into left femoral artery. Rapid epithelization of most of the ulcerated areas and marked prompt healing of leg lesions.

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PLATE 96

FIGURE 5.—Intra-arterial catheter in right internal mammary artery with Diodrast outlining the course of this vessel and the rich vascular supply to the large metastatic mass overlying the sternum.

FIGURE 6.—Ultraviolet photograph. Outline of distribution of right internal mammary artery following injection of fluorescein into catheter at position in figure 5. Note supply to right half of tumor mass on the sternum.

FIGURE 7.—Ultraviolet photograph. Fluorescence of distribution of thyrocervical axis with catheter inserted into this vessel.

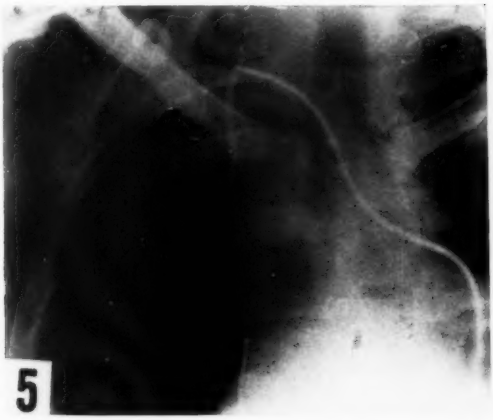
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PLATE 97

FIGURE 8.—Erythema and serum formation beneath epidermis within 24 hours after HN2 injected into thyrocervical axis.

FIGURE 9.—Slough well advanced in area of early bullous formation (fig. 8) and developing within the distribution of arteries injected 28 days after HN2. Note decrease of right half of sternal mass.

FIGURE 10.—Slough advanced in all areas with increase of tumor mass, particularly from the left, 69 days after intra-arterial HN2.

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THE PREPARATION AND HANDLING OF REPLICATE TISSUE CULTURES FOR QUANTITATIVE STUDIES[1]

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INTRODUCTION

In determining the effect of various agents on cell or nuclear proliferation and disintegration in tissue culture, differences in response are accurately measurable only when replicate tissue cultures can be planted in large numbers and grown with minimal loss of cells at each renewal of culture fluid. The lack of a procedure for obtaining such large numbers of replicate cultures has limited the use of tissue cultures in quantitative studies. With the recent development, however, of a cell strain originated from a single cell (*1*), and surface substrates, such as cellophane (*2*, *3*), massive growth of a pure strain of cells is available as a source of large volumes of cell suspension for planting these replicate cultures.

This paper describes the method for preparing and handling replicate cultures and the experimental development and validation of this method.

MATERIALS AND PROCEDURES

The equipment used in the preparation and handling of replicate cultures is described below:

Culture flasks, designs T–12 and T–60 (*4*). Rake and spatula, shown in text-figure 1 A, B, C, D. The handles were drawn from glass tubing, and the shafts were commercially available hardened platinum alloy (15% iridium).[3] The tip of each was shaped with a small ball pien hammer, and the edge smoothed under a low-power microscope on a knife-blade hard Arkansas oil stone.[4]

Centrifuge, International, fitted with a #757 tachometer[5] and head #277 to carry 250-ml. tubes #384.

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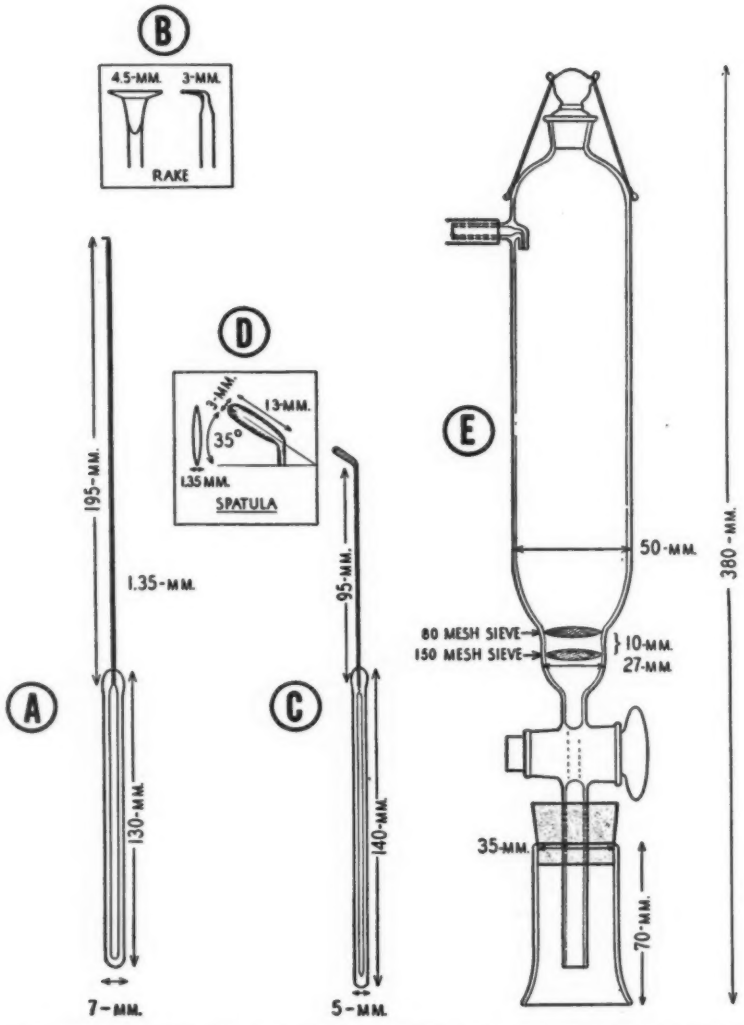
[1] Received for publication October 17, 1950.
[2] The authors are indebted to Mr. Marvin Schneiderman of the Biometrics Section, National Cancer Institute, for his statistical evaluation of the data, and to Mr. Frederick Highhouse, Head of the Instrumentation Unit, for his assistance in the design and for the fabrication of the special glass apparatus used.
[3] Baker & Co., Inc., 13 Astor St., Newark 5, N. J.
[4] Behr-Manning, Division of Norton Co., Troy, N. Y.
[5] International Equipment Co., Boston, Mass.



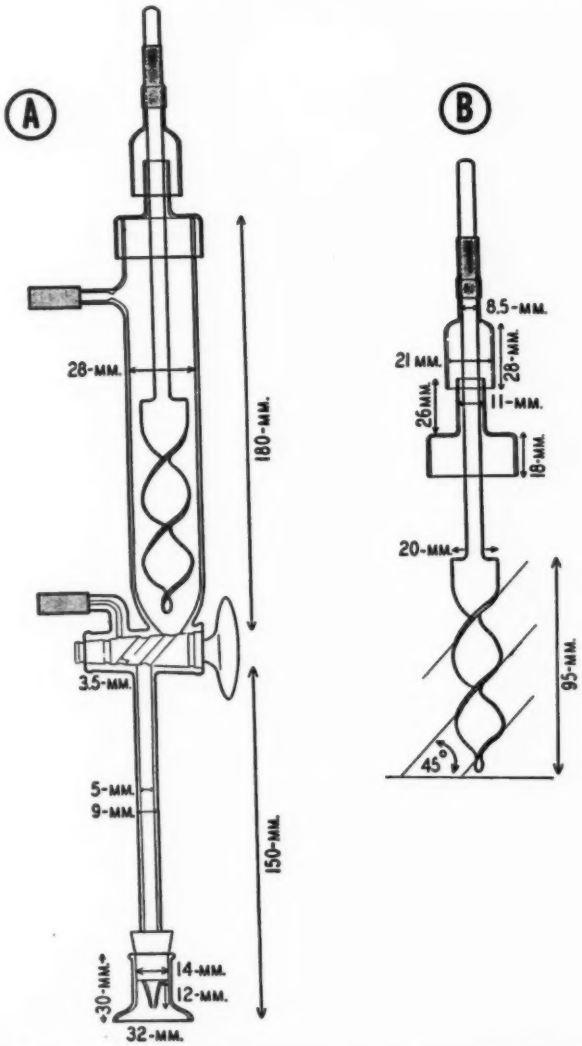
TEXT-FIGURE 1.—Equipment for preparing cell suspension. A, rake for scraping cells from cellophane and glass substrate of T-60 flasks; B, insert showing detail of rake tip; C, spatula for scraping cells from cellophane and glass substrate of T-12 flasks; D, insert showing detail of spatula tip; E, sieve unit for sieving cell suspensions.

Sieve unit, shown in text-figure 1E. The wire in the 80-mesh sieve was the commercially available hardened platinum alloy (10 percent rhodium) with a wire diameter of 0.003 inches; that in the 150-mesh sieve was the commercially available platinum alloy (1 percent iridium)[6] with a diameter of 0.0016 inches. The sieves were fused into the glass shell and the unit oven annealed for approximately 12 hours.

[6] American Platinum Works, Newark 5, N. J.

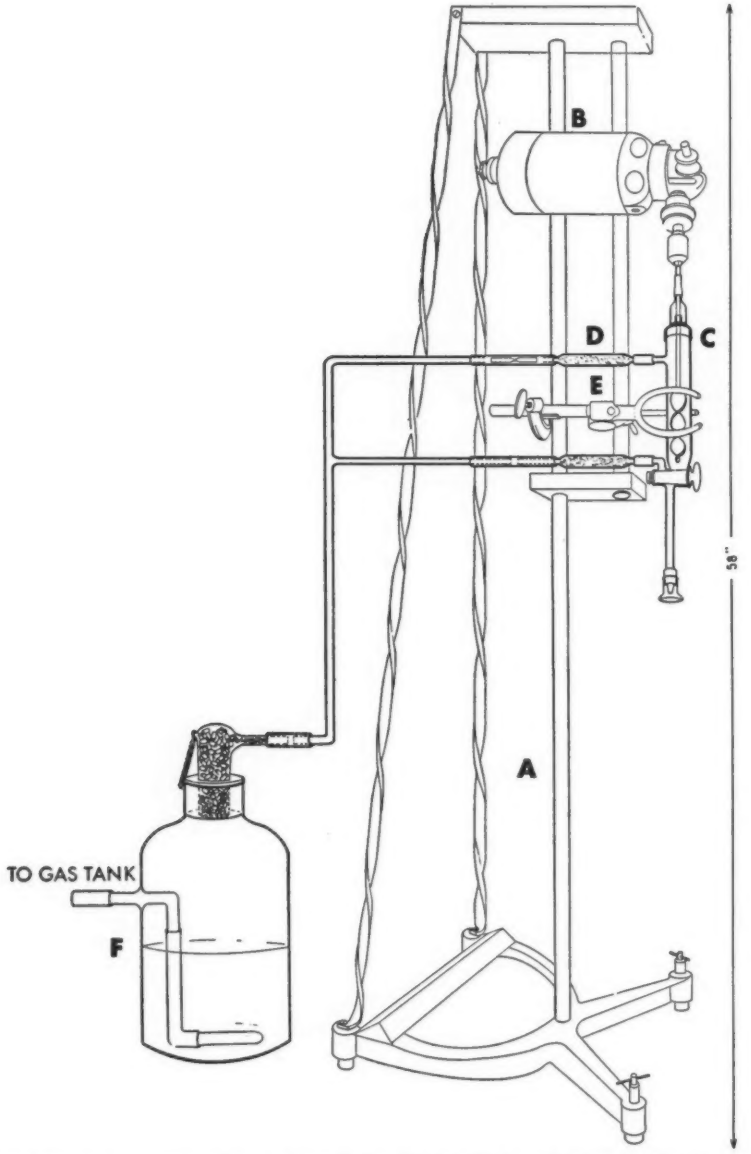
White petrolatum, U. S. P., used as stopcock lubricant, autoclaved in 1.0-ml. or 2.0-ml. lots in glass tubes, with a wooden applicator inserted in each. Each tube was autoclaved and used only once.

Reservoir for cell suspensions, shown in text-figures 2 and 3C. This pyrex reservoir was designed with a broad base at the stopcock junction, special 3-way stopcock with a gas inlet, and tip calibrated to deliver 1 ml. (±0.01). The reservoir top was covered with a glass reservoir cap (text-fig. 2). The bores of the stopcock plug were 3.5 mm. in diameter. The plug had a shallow circumferential groove cut into



TEXT-FIGURE 2.—Reservoir and stirrer assembly. A, reservoir for containing cell suspensions during the interval of stirring and delivering cells as replicate samples to flask; B, glass stirrer for stirring cells in the reservoir.

it near each end. A sleeve attached by a cork to the delivery tip was designed to protect the delivery tip from contamination and to guide and center the T–12 flask.



TEXT-FIGURE 3.—Arrangement of apparatus for dispensing cell suspensions. A, support stand; B, electric stirrer; C, reservoir assembly; D, gas trap; E, versatile clamp; F, gas humidifier.

Stirrer, glass shown in text-figure 2. The blade of the glass stirrer was fabricated from a strip cut from the bottom of a 6-inch diameter pyrex petri dish. The blade was fused to a glass rod. A glass sleeve was attached by rubber tubing to the glass rod which was slipped through the reservoir cap.

Stirrer, electric motor assembly, heavy duty, variable speed, governor type,[7] shown in text-figure 3B.

Tachometers,[8] Stewart-Warner portable indicating models, with range of 50 to 5,000 r. p. m.

Support stand shown in text-figure 3A. A wide-base stand[9] fitted with levelling screws was modified by substituting a 50-inch-long rod of 1-inch diameter for the original vertical support rod. Spirally wound, diagonal stabilizing straps, ¼ by ½ inch, were added to connect the base with a horizontal plate topping the upright bar. The base was weighted with a lead triangular cross bar.

Versatile clamp, three prong grip[10] shown in figure 3E.

Clamp holders,[11] size to fit 1-inch diameter rod shown in figures 3 and 5.

Tank of gas mixture,[12] 5% CO_2 in air, of 220 cu. ft.

Hoke-Phoenix two-stage reducing valve, for oxygen,[13] with a maximum delivery pressure gauge of 10 p. s. i.

Gas humidifier, shown in text-figure 3F. The humidifier consisted of a 2-liter pyrex bottle with side arm inlet of glass tubing cemented to a Selas XFF filter.[14] A spray trap shown in text-figure 3F, with side arm outlet was securely wired in the ground glass neck of the bottle. The spray trap was fitted at the bottom with a perforated glass disk and was packed with short lengths of glass tubing held in by a folded wadding of stainless steel wire. The bottle contained 1% NaCl solution.

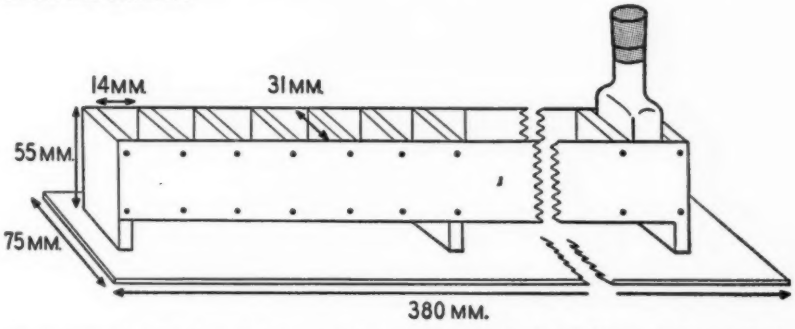
Gas, traps, shown in text-figure 3D. These were packed with sterile cotton. A gas reducing capillary tube was inserted in the gas inlet line at the point of attachment to the reservoir gas trap.

T–12 flask rack, shown in text-figure 4.

Rectangular porcelain support stand,[10] shown in text-figure 5A.

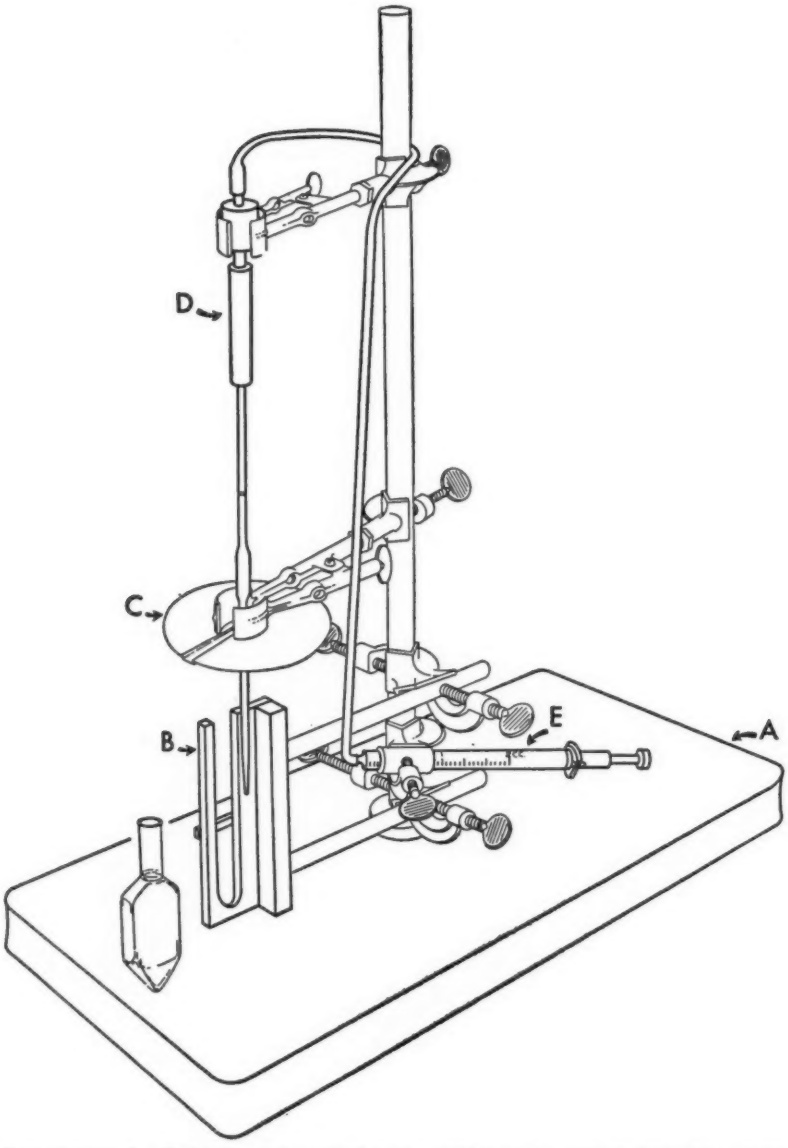
Flask-centering frame, shown in text-figures 5B and 6.

Pipette-positioning clamp and shield, shown in text-figure 5C. This clamp was designed from a self-closing spring clamp[7] to each jaw of which a semicircular metal shield was attached.

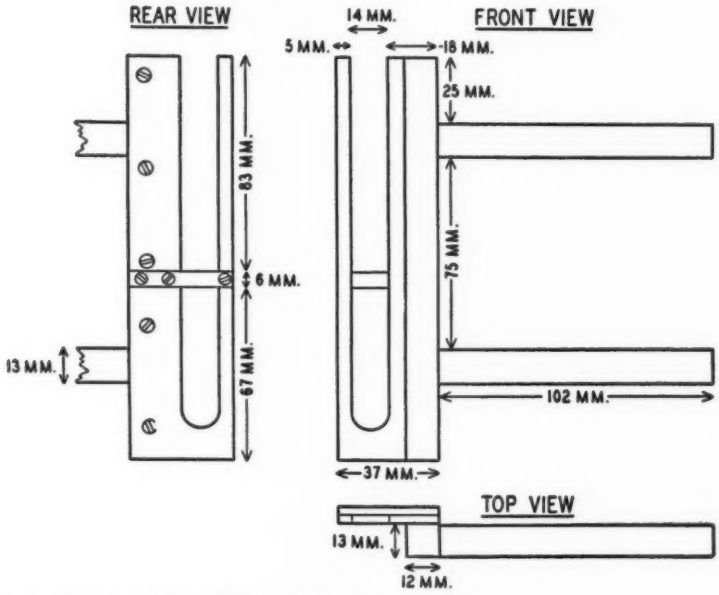


TEXT-FIGURE 4.—Flask rack for supporting T–12 flasks during fluid-renewal procedure.

[7] A. S. Aloe Co., St. Louis, Mo.
[8] Carey Machinery and Supply Co., 119 E. Lombard St., Baltimore 2, Md.
[9] American Instrument Co., Silver Spring, Md.
[10] Eimer and Amend Co., New York, N. Y.
[11] Central Scientific Co., Newark 8, N. J.
[12] Southern Oxygen Co., Washington, D. C.
[13] Hoke Inc., Englewood, N. J.
[14] Selas Corporation of America, Philadelphia 34, Pa.



TEXT-FIGURE 5.—Fluid-removal assembly for withdrawing supernatant fluid from T–12 flasks. A, rectangular porcelain support stand; B, flask-centering frame; C, pipette-positioning clamp and shield; D, rubber coupling for pipette; E, 2-ml. syringe.



TEXT-FIGURE 6.—Detail of flask-centering frame.

Volumetric pipettes to deliver 1 ml. and 100 ml at 20° C.
Rubber coupling for pipette, shown in text-figure 5D.
Self-closing spring clamp for supporting rubber coupling, shown in text-figure 5.
Syringe, 2 cc., shown in text-figure 5E.

PREPARATION OF CELL SUSPENSIONS

Cells used for the preparation of suspensions were grown in T–60 flasks (*4*) under OC perforated cellophane (*5*) in 8 to 10 ml. of 40 percent horse serum, 20 percent (1:1) embryo extract, and 40 percent balanced saline (*6*). Each parent culture was planted from approximately ½ of the cells scraped with the platinum rake (text-fig. 1A) from a stock T–60 culture. Cultures were grown for 10 to 14 days so that the parent cultures used for planting the replicate cultures showed a cell density comparable to that shown in figure 1. This density represents approximately 3,000,000 nuclei per ml. of culture fluid. Cultures were selected which showed few or no areas of necrotic cells.

Following removal of the culture fluid, the cells of the parent cultures were washed with several rinses of fresh culture fluid and then scraped from the upper and lower surfaces of the cellophane and from the glass floor of the flask with the platinum rake (text-fig. 1A). The cell suspensions were pooled and centrifuged at 1,100 r. p. m. for 15 minutes. The supernatant was removed by pipette and discarded, and the packed cells were resuspended in a volume of fluid calculated to yield a concentration of cells higher than that finally desired. The suspension was transferred by

means of a 100-ml. pipette to the sieve unit (text-fig. 1E), and was allowed to flow through the sieves by force of gravity. Occasionally, to accelerate the flow of cell suspension through the sieve, the humidified gas mixture at a pressure of 1 lb. per square inch—that pressure which never forced the cell suspension through the sieve in a damaging jet—was admitted through the side-arm inlet of the sieve. The trap of sterile cotton placed at the inlet served as a bacteriological filter for this gas. The sieved suspension was collected into a sterile flask. In subsequent transfer to the reservoir the total volume of suspension was measured.

Stirring and Gassing of Cell Suspension

The glass stirrer was inserted in the reservoir, which was then mounted in the versatile clamp on the stand supporting the motor for operating the stirrer. The shaft of the glass stirrer was clamped in the chuck of this electric stirrer motor (text-fig. 3). The design of the assemblage is shown in text-figures 2 and 3.

To assure uniformity of the cell suspension it was stirred in the reservoir at 450 r. p. m. continuously during sampling and for at least 15 minutes before sampling. During the continuous stirring, the cell suspension was gassed through the side inlet in the reservoir with a slow flow of the gas mixture. Before entering the reservoir the gas mixture was saturated with water vapor by passage through the humidifier. The Selas XFF filter submerged in the saline served to disperse the gas, and the spray trap served to eliminate water droplets from the gas mixture. The volume of gas entering the reservoir was reduced by a capillary stricture in the inlet line. The trap of sterile cotton placed between the stricture and the reservoir inlet served as a bacteriological filter for the gas.

Standardization of Cell Suspension

One-milliliter samples were drawn from the stirred suspension for determining the concentration of cells by enumeration of their nuclei. Each sample was discharged from the delivery tip of the reservoir by a jet of the gas mixture at a pressure of about 2 lb. per square inch. The gas was jetted into the delivery tip through the gassing inlet of the three-way stopcock; this inlet was also protected by a sterile cotton trap. In charging the delivery tip with cell suspension from the reservoir, some of the suspension was lost from the tip; this excess was caught in a waste receptacle, and the volume measured.

The number of cell nuclei in the 1-ml. samples was estimated by the nuclear count procedure (7). The volume of cell suspension left in the reservoir to be diluted was calculated by subtracting the volumes discharged in the sampling from the total originally introduced. From these data the volume of diluting fluid necessary to yield the desired concentration of cells was calculated. This volume was introduced by a graduated pipette through the top into the reservoir.

Inoculation of Culture Flasks

The culture flasks used in this study were of the T–12 design. A square of OC perforated cellophane 3.8 cm. to a side and cleaned as previously described (*5*) was inserted into each flask, moistened with triple distilled water, and molded to the floor and sides by means of a platinum spatula (text-fig. 1). Then the flasks were drained, plugged with cotton, and autoclaved.

Prior to introduction of the cells into a flask, 1.5 ml. of the fraction of culture medium not used in suspending the cells was introduced into the flask. At this time if cellophane sheets were disarranged on the floors of any of the flasks they were realigned with the platinum spatula. Each flask was then inoculated with 1 ml. of stirred suspension ejected from the reservoir delivery tip into the flask by the gas. The gas mixture used to eject the cell suspension was allowed to flow for 30 seconds to replace the air in the flask with the 5 percent carbon dioxide in air mixture. For this inoculation of the flask the portion of the delivery tip protected by the glass sleeve was long enough to project well into the culture flask and so prevented spattering of cells or fluid on the neck of the flask when the cell suspension was discharged. Following gassing, the flask was quickly sealed with a size 00 rubber stopper and was incubated in horizontal position at 37.5° C.

Renewal of Culture Fluid

In the culture fluid renewal, made three times weekly, flasks were centrifuged at 1,100 r. p. m. for 20 minutes. After centrifugation, the flasks were transferred with a minimum of agitation to the flask rack (text-fig. 4) and the supernatant on each flask was withdrawn. In withdrawing the culture fluid, a sterile 1-ml. volumetric pipette was used for each flask. The pipette was inserted into a short piece of rubber tubing, part of the rubber coupling (text-fig. 5D) clamped in vertical position, and then the pipette was adjusted in the pipette-positioning clamp. The flask, placed in the centering frame (text-fig. 5B) during the removal of supernatant fluid, was slowly raised in the frame, which guided and steadied the flask, until the tip of the positioned pipette was just submerged in the culture fluid. The vertical slot in the centering frame facilitated observation of the submerged pipette tip. The plunger of the syringe was withdrawn slowly to raise the fluid into the pipette and up to the 1-ml. calibration. Occasionally between consecutive fluid removals, the shield of the pipette positioning clamp (text-fig. 5C), which served to protect the mouth of the flask, was given a flash-flaming.

After withdrawal of the fluid the cells packed by the centrifugation were resuspended by vigorous tapping of the flask by hand and the sheets of cellophane that had been disarranged were realigned with the platinum spatula. One milliliter of culture fluid was added from the cell-suspension reservoir used without the stirrer for fluid renewals. The flask was gassed through the gas inlet of the three-way stopcock, quickly sealed with the 00-size rubber stopper, and returned to the incubator.

EXPERIMENTS AND RESULTS

Physiological Effect of Stirring Cells in Suspension

Introduction

For preparing replicate cultures, cells in suspending fluid must be stirred at a rate sufficient to yield a uniform suspension. Since too rapid agitation can injure cells, it was imperative in the development of this procedure to determine experimentally with the strain of cells used whether stirring cells in culture fluid might so injure the cells that the subsequent proliferation might be affected.

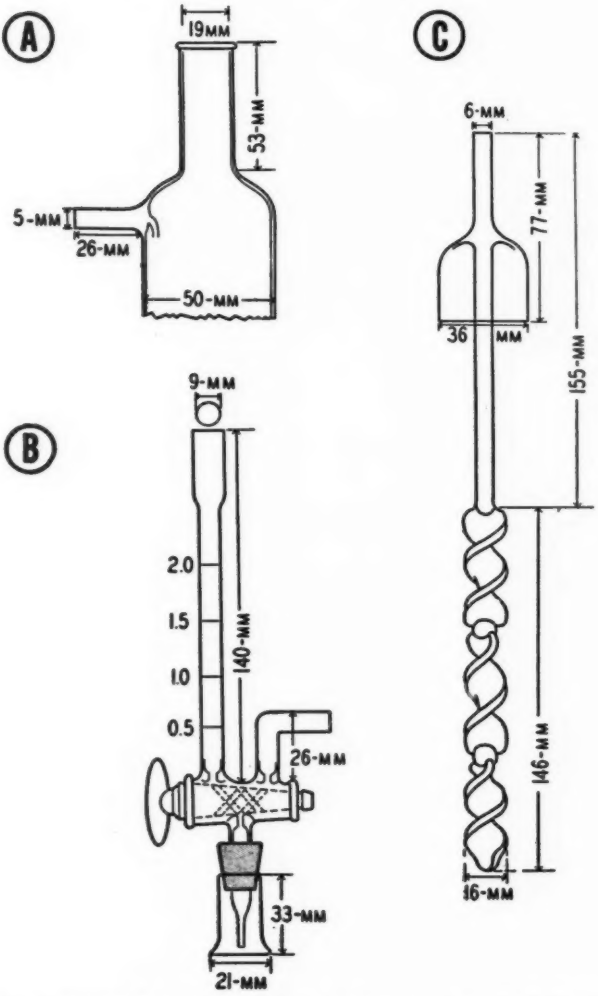
Experimental

Control cultures were prepared from cell suspensions of strain L and were stirred for the minimal period of 15 minutes. Experimental cultures were planted from the same cell suspensions subjected to additional periods of 1 and 2 hours of continuous stirring. To compare the proliferation of control and experimental cultures, cultures were grown in some instances for 72 hours, in others for 10 days, after which the numbers of cell nuclei per flask were determined (7).

In the early experiments on the effect of stirring on the cells the method of preparing the cultures was identical to that previously described, except that certain units of equipment were used which were later altered in developing the final procedure as given in this paper. For instance, in the earlier experiments the cell suspension was passed through a single 80-mesh sieve, and the gas humidifier varied slightly in design; the apparatus was functionally similar to that previously described. Also, the reservoir consisted of a modified tubular glass unit 50 mm. in diameter with a separate burette calibrated in 0.5 ml. to deliver 2-ml. samples of cell suspension (text-fig. 7). The glass stirrer with this reservoir assembly for these early experiments had 2 spiral turns at a pitch angle of 55° to 58° and a blade diameter of 12 mm. In 4 preliminary experiments the cells were stirred with this stirrer at 425, 710, and 1,400 r. p. m., and were grown for 2 to 4 weeks to determine whether or not continuous stirring at the higher speeds damaged them. In a group of later, more detailed experiments the cells were stirred at 950 r. p. m. in the same reservoir with a glass stirrer that had 7 spiral turns at a pitch angle of 35° and a blade diameter of 16 mm. (text-figs. 7 and 8).

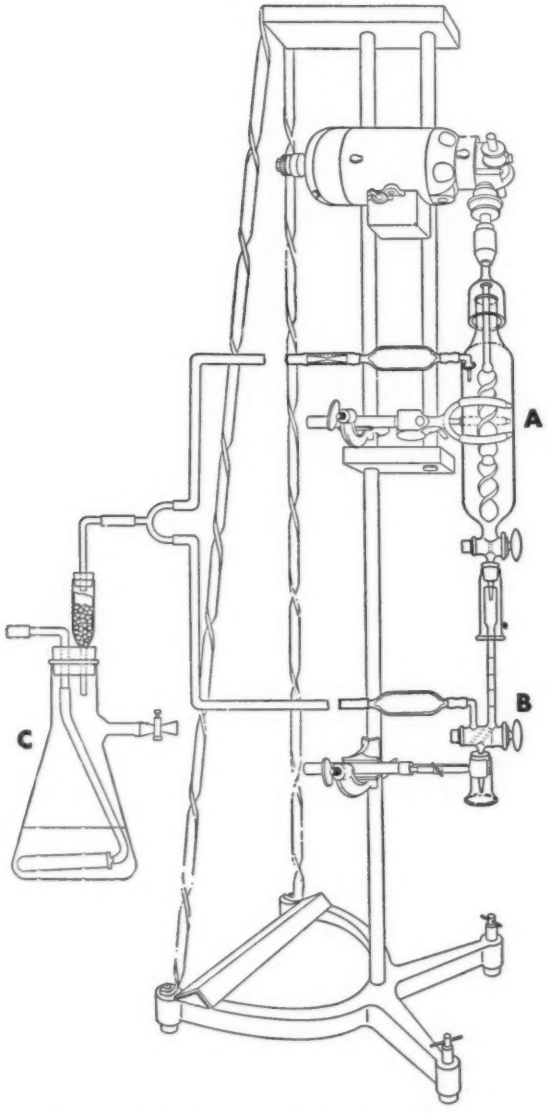
Results and Conclusions

The results of the preliminary study on the effect of stirring the cells indicated that at 425, 710, and 1,400 r. p. m. no recognizable cell damage occurred; rapid proliferation took place in the subsequent 2- to 4-week growth period. The results of the later more exhaustive study with the second design of stirrer are summarized in text-figure 9. The numbers of cell nuclei in the cultures at the end of 72 hours' and 10 days' growth were



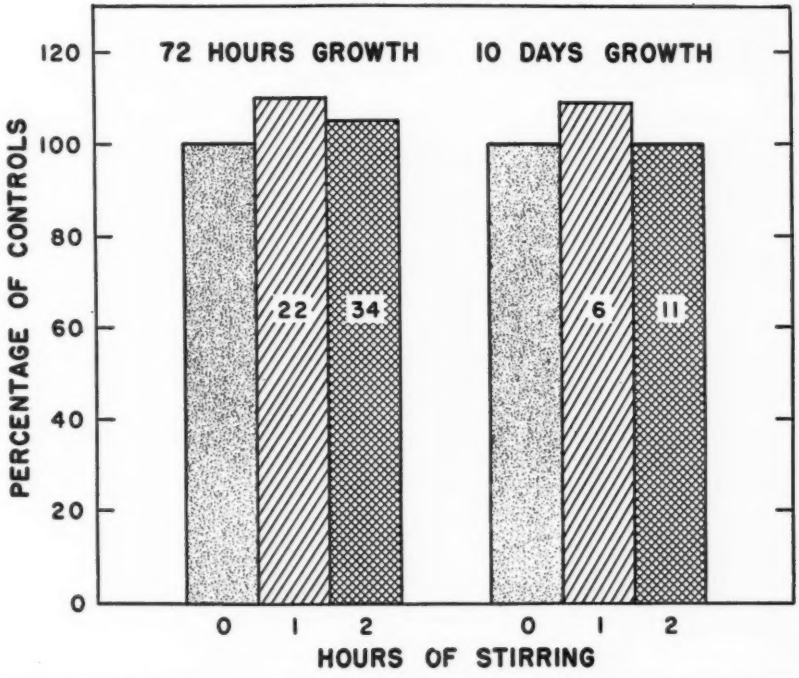
TEXT-FIGURE 7.—Detail of reservoir, stirrer, and separate burette of early assembly. A, top of reservoir; B, separate burette for dispensing stirred cells; C, stirrer for stirring cells in the reservoir.

estimated by the determination of the numbers of nuclei (7) per culture. The experimental cultures were compared with their respective control cultures. The average number of nuclei per experimental culture was expressed as a percentage of the average number of nuclei per control culture. The number of experimental cultures used for each determination is indicated in text-figure 9. Control cultures identical in number with the experimental cultures were used for each comparison.



TEXT-FIGURE 8.—Arrangement of apparatus for dispensing cells, early equipment. A, funnel and continuous leaf stirrer; B, separate burette; C, gas humidifier.

The data indicate that cells continuously stirred for 1 hour or even for as long as 2 hours had grown as well at the end of 72 hours and 10 days as the control cells not subjected to the prolonged stirring. The apparent increase in proliferation of cells stirred for 1 hour (text-fig. 9) was statistically nonsignificant, since in using this early design of equipment there



TEXT-FIGURE 9.—Influence of stirring on cell proliferation; cells stirred in culture fluid.

was sufficient variation between individual cultures to account for this rise simply on the basis of chance. Irrespective of speed and time of stirring, within the range studied there was no evidence of any cell injury.

EFFECT OF FLUID-CHANGE PROCEDURE ON CELL LOSS

Introduction

By the conventional method of removing exhausted nutritive fluid from tissue cultures, loss of cells occurs. Since both these cells and their potential descendants are lost, the rate of proliferation that is measured is not a reliable index of the cell proliferation which would have occurred in cultures planted as replicates. An important step in the development of the quantitative procedure was the elimination of this variable and considerable cell loss. Since light centrifugation of the cultures before removal of the supernatant fluid seemed feasible, procedures were tested to determine the optimum speed and time of centrifugation as well as the most efficient pipetting method for minimal cell loss.

Experimental

Sets of replicate cultures of the pure strain of cells originated from a single cell of the strain L were planted and grown for 4 weeks. The cul-

ture fluid on each flask was renewed three times a week. In this removal of culture fluid, several procedures were tried.

The influence of speed of centrifugation of the culture flasks on clearing of the supernatant fluids and on subsequent proliferation of the cells was tested. Three sets of cultures were centrifuged at 1,100, 1,400, and 1,800 r. p. m., respectively, for 20 minutes three times a week, for a period of 3 weeks. The over-all changes in cell nuclei numbers after the 3-week period were determined by the enumeration of nuclei in aliquots of the culture (*7*).

A comparative study was made of the effectiveness of 10- and 20-minute periods of centrifugation at 1,100 r. p. m. in clearing the supernatant fluids of the cultures.

Two methods for the removal of the supernatant fluids were compared. In the first, the flask was held by hand while a 1-ml. volumetric pipette connected by rubber hose to a mouth piece was introduced into the upper fluid layer. The fluid was withdrawn by mouth suction. The second method was that already described in which a mechanical guide (text-fig. 5) was introduced for positioning the pipette and guiding and supporting the flask.

Results and Conclusions

Although no apparent cell damage resulted from the repeated centrifugation of cultures at speeds up to 1,800 r. p. m., the two higher speeds of centrifugation caused disarrangement of the sheets of cellophane and occasional breakage of flasks. There was also a tendency to produce too tightly packed pellicles of cells in the tip of T–12 flasks. For these reasons the speed of centrifugation was limited to 1,100 r. p. m.

The data from the quantitative experiments testing the effect of time of centrifugation and methods of fluid removal on loss of cell nuclei in the supernatant are summarized in table 1. At each fluid renewal, the removed fluids from all flasks of an experimental set were pooled and the average loss per flask was determined by the enumeration of nuclei (*7*). Since there were 3 fluid renewals per week, the weekly cell loss per flask represented the sum of the 3 average-per-flask determinations. The weekly cell loss per flask was expressed as the percentage of the average number of cell nuclei present in the flask at the close of that week. The percentages in table 1 were averages of 1 or more determinations. Each determination was based on 1 experimental set comprising 3 to 12 flasks.

The data from the first week of culture are separated in table 1 from data of later weeks because directly after planting cells tend to be less well anchored to the substrate and therefore greater cell loss might occur. Since the number of cells present in the culture might influence the percentage of cell loss, the data from cultures containing fewer than 1,000,000 nuclei are separated in table 1 from those of cultures containing more than 1,000,000 nuclei.

TABLE 1.—*Loss of cells from changing supernatant fluids*

Procedure		Average weekly cell loss in percentage of total cells per flask							
		Number of nuclei in flask							
		First week				After first week			
		Fewer than 1,000,000		More than 1,000,000		Fewer than 1,000,000		More than 1,000,000	
Centrifugation time in minutes	Method of removal	Number of determinations	Percentage	Number of determinations	Percentage	Number of determinations	Percentage	Number of determinations	Percentage
10	Hand	1	6. 45	3	0. 81	------	------	7	1. 13
20	Hand	4	1. 48	2	0. 90	1	0	11	0. 34
20	Mechanical	5	0. 60	7	0. 23	1	0. 10	25	0. 07

The data of table 1 show a trend toward reduced cell loss with the increase in time of centrifugation from 10 to 20 minutes. The introduction of the mechanical method of supernatant removal further reduced the cell loss to values ranging from 0.10 to 0.60 percent for cultures containing fewer than 1,000,000 nuclei, and to values ranging from 0.07 to 0.23 percent for cultures containing more than 1,000,000 nuclei.

It is concluded from this study that with a centrifugation speed of 1,100 r. p. m. for 20 minutes, and with the mechanical method of fluid removal, the loss of cell nuclei at the fluid renewals is insignificant.

EFFECT OF APPARATUS DESIGN ON ACCURACY OF PREPARING REPLICATE CULTURES

Introduction

The accuracy of this procedure is dependent on the degree of replicacy of the samples obtainable from the cell suspension. Since for replicate sampling, a cell suspension must be uniformly dispersed and equally distributed to the culture flasks, numerous designs of apparatus for preparing, stirring, and dispensing cell suspensions were developed and experimentally tested. The designs of apparatus tried and the results of the experimental tests are described in this study.

Experimental

Sieve design.—The first design of sieve unit used consisted of the 80-mesh platinum-rhodium sieve fused into a glass tube. The cell suspension was drawn into a pipette and jetted through the sieve under the pressure effected by blowing through the pipette as its tip was held 2 or 3 cm. above the sieving.

The second design of sieve was the one previously described and ultimately adopted for use (text-fig. 1). This sieve unit contained both the 80- and 150-mesh grating through which the cell suspension was slowly

passed by either the force of gravity or less than 1 pound positive gas pressure. In most instances no gas pressure was necessary.

Cell suspensions prepared with both single and double sieves were stirred and sampled. The nuclei in the samples were counted (7) and the variability among the samples was determined. Sample cultures from the sieve suspensions were also grown for periods of from 1 to 4 weeks to compare the numbers of nuclei.

When the cell nuclei of samples from the sieved and stirred suspensions were counted, the data collected suggested a tendency toward greater uniformity of cell distribution in the suspensions passed through the double sieve. Sample cultures of these suspensions appeared to grow as well as did those of suspensions passed through the single 80-mesh sieve.

Stirrer design.—Two designs of electric motor stirrers were used: a light-duty induction motor stirrer [9] and the variable speed governor-type electric stirrer previously described in the procedure. Chucks were fabricated for the induction-motor stirrer, and certain of these stirrers were also altered by the addition of a reduction-pulley system, which lowered the speed to about 800 r. p. m.

The light-duty stirrer was found to have too light a motor for operating the glass stirrer at uniform speed. This motor was therefore replaced by the heavier governor-type motor, which operated the glass stirrer at more constant speed. Although this variable-speed motor was used in the final procedure outlined in this paper, the motor required constant checking of the setting, and the shaft was not entirely free of wobble. The use of a constant-speed motor with shaft connected by gear or pulley and belt to a second shaft supported on separate bearings and fitted with a small size precision Jacobs chuck [8] for holding the glass stirrer, so that its free end has a minimum of whip, might obviate these difficulties.

Glass stirrer design.—Three screw-type designs of glass stirrers were experimentally tested. Observations were made on the extent of frothing of the cell suspensions, the degree to which cells settled in the suspension, and the eddying of the fluid-suspension medium when all these designs of stirrers were used with their respective reservoirs. The first stirrer had 2 spiral turns, with a pitch angle of 55–58°, and a diameter of 12 mm. The second (text-figs. 7 and 8) had 7 spiral turns, with a pitch angle of 35°, and a diameter of 16 mm. The first 2 designs of stirrer were tested in a reservoir with an internal diameter of 50 mm. The third design (text-figs. 2 and 3), the one finally adopted, having 3 spiral turns with a pitch angle of 45–50°, was tested in a reservoir of 28 mm. internal diameter. All stirrers were tested at the subsequently designated speeds to determine the optimum speed for uniform distribution.

Cell suspensions were stirred with the first design of stirrer at speeds of 450, 950, and 1,700 r. p. m. In early studies the second design of stirrer was tested at 450, 950, and 1,700 r. p. m. In the later extensive cell-enumeration studies with this stirrer a speed of 950 r. p. m. was used. The third design of stirrer was tested at both 350 r. p. m. and 450 r. p. m. In testing the uniformity of cell distribution in the cell suspension, samples

of equal volume were drawn from the suspensions while they were being stirred. The numbers of cell nuclei (7) in each sample were determined; the relative variation among samples was calculated.

With both the first and second designs of stirrer where the ratio between diameters of the stirrer and the reservoir was wide, the two higher speeds of stirring were more effective in producing uniform distribution of cells. The first design required such increased velocities of rotation to keep the cells suspended in the upper layers of fluid that excessive amounts of frothing resulted. Even with the high velocities of stirring the distribution of cells as measured by variation between samples was not uniform. At the speed of 1,700 r. p. m. with the second design of stirrer the excessive frothing reduced the quantity of fluid suspension so that insufficient amounts of cell suspension remained for planting cultures. For this reason a velocity of 950 r. p. m. was selected as the highest speed practical with the second stirrer, but with this speed some slight frothing occurred.

The results of the experiments testing the uniformity of cell distribution in suspensions stirred with the second design of stirrer are presented in table 2. The relative variation or coefficient of variation was calculated for sample inocula drawn from 7 suspensions. Three to 6 flasks were inoculated from each suspension. The relative variations ranged from 1.1 to 16.2 percent.

TABLE 2.—*Accuracy of planting procedure*

Average inoculum size in numbers of nuclei per flask	Number of flasks	Relative variation in percent		Average inoculum size in numbers of nuclei per flask	Number of flasks	Relative variation in percent	
		Procedure 1[1]	Procedure 2[2]			Procedure 1[1]	Procedure 2[2]
244,000	3	10.8		730,000	5		2.1
275,000	6	12.4		825,000	6		7.1
351,000	6		10.4	1,017,000	6	16.2	
458,000	3	7.4		1,036,000	3	1.1	
502,000	5		6.3	1,196,000	5		6.2
601,000	6		5.0	2,093,000	3	3.5	
602,000	3		2.9	4,394,000	3		2.0
715,000	5	5.4		5,483,000	3		2.4

[1] Procedure 1: Use of second design of stirrer (text-fig. 7) with first design of funnel (text-figs. 7 and 8).
[2] Procedure 2: Use of third design of stirrer (text-fig. 2) with third design of funnel (text-figs. 2 and 3).

With these first two designs of narrow-diameter stirrer, high speeds of rotation were required for uniform distribution of the cells because with the lower speeds the stirred fluid tended to be agitated near the center axis of the reservoir while peripherally the cells settled out of suspension. Since high speeds of stirring tended to produce frothing and could theoretically have injured cells, although no cell injury was observed, the third stirrer was designed with a greater diameter while the reservoir with which it was used was of smaller diameter, thus reducing the clearance between the stirrer and reservoir and giving more effective stirring at the reduced speeds of 350 and 450 r. p. m. Data from the sampling of 9 suspensions stirred with this design of stirrer are tabulated in table 2.

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The relative variation or coefficient of variation of the samples from each suspension ranged from 2.0 to 10.4 percent.

Reservoir design.—In the early experiments the reservoir consisted of 2 units: a modified tubular reservoir and a separate burette. The tubular glass reservoir had a side arm inlet and throat altered for insertion of the stirrer (text-figs. 7 and 8). The burette was calibrated in 0.5 ml. to deliver 2.0 ml. For the complete evacuation of the burette tip, gas was jetted through the gassing inlet of the three-way stopcock (text-figs. 7 and 8). This design reservoir was used with the first and second glass-stirrer designs.

A second reservoir model was designed with the bore of its stopcock plug enlarged to deliver 1 ml., and this volume of cell suspension was completely ejected from the enlarged stopcock bore by jetting of gas through the gassing inlet of a third orifice. This reservoir, 28 mm. in diameter, was specially fabricated with a wide throat for insertion of the third design of stirrer and had a base broadened at the stopcock junction.

The third reservoir model and the one used in the final procedure was also designed with the burette integral as shown in text-figure 2. The reservoir outlet tube was elongated and calibrated to deliver 1 ml. The protective sleeve shielding the delivery tip was improved for guiding and centering both T–12 and T–60 flasks. The stopcock plug was circumferentially grooved at each end and was lubricated with white petrolatum peripheral to these limiting grooves so that no vaseline worked into the bore of the burette to interfere with its exact calibration. The central portion of the plug was thus lubricated by the suspending medium alone.

Several difficulties were encountered in using the first design of reservoir. Cells tended to settle in the narrow constricted base of the reservoir before stirring. The cells were thus difficult to resuspend. In the stopcock bore and delivery tip of the reservoir where the fluid was not agitated by the stirrer, cells settled out of suspension. If this reservoir outlet tube exceeded in capacity the volume of suspension to be delivered to the burette, unequal cell sampling in consecutive burette fillings could occur because the cells settled in the tube so rapidly that the fluid left in the tube of the reservoir was free of cells. Even when the reservoir outlet tube was reduced in volume, slow filling of the burette from this unstirred source of suspension introduced uncontrollable variations in numbers of cells in each measured volume, due to settling of the cells. When rapid fillings of the burette were made in an attempt to compensate for this settling there was difficulty because any overcharging beyond the calibration mark necessitated discarding the entire sample. This design of reservoir was used to dispense samples in experiments summarized in table 2.

The second design of reservoir proved entirely unsatisfactory. In calibrating the enlarged stopcock bore, though designed to deliver 1.0-ml. samples, no uniformity of sample volume was obtained.

The third design of reservoir, the one ultimately chosen, was used to dispense samples in the experiments previously mentioned and summa-

rized in table 2. The relative variation of samples of 3 to 6 flasks inoculated from 9 cell suspensions stirred with the broad blade stirrer and dispensed from this type of reservoir varied from 2.0 to 10.4 percent. Of these 9 sets of samples, all but 1 had a relative variation of less than 10 percent and 5 sets had a relative variation of 5 percent or less. These results indicated not only a reduced coefficient of variation for each experiment but also a greater uniformity between experiments in the variations obtained.

Results and conclusions

The relative variation in percent divided by the square root of the number of flasks in a set gives the 95 percent limits of the experimental error of the procedure, so that with the equipment and procedure ultimately selected and described the experimental error of the procedure finally developed in this study is less than 5 percent.

DISCUSSION

The procedure for preparing and handling replicate cultures given in this paper is of fundamental usefulness in comparative quantitative studies on the physiology of tissue-culture cells when these cells are subjected to a variety of experimental conditions. Although the procedure has been validated in the phases presented with the single cell strain of strain L, the procedure should be equally applicable or readily adapted to any strain of cells that will grow on cellophane or other surface substrates and that can be scraped or shaken from this substrate to form a suspension.

The data of this study have demonstrated no apparent physiologic damage to the cells resulting from sieving and stirring them in a culture fluid made up of horse serum, chick embryo extract, and balanced saline when this suspension was continuously gassed to adjust and maintain the pH at approximately 7.3. These cells on subsequent culturing continued to proliferate actively as indicated by continued increase in the numbers of nuclei. These cultures could be grown without significant losses of cell nuclei at each fluid renewal. The light centrifugation of the cultures necessary to clear the supernatant fluid of cells appeared not to affect their subsequent proliferation rate. This system of fluid renewals allowed rapid processing without contaminations of large numbers of cultures.

With this procedure, at least 100 replicate cultures may be prepared by 2 operators in a period of 4 hours. The experimental error of the planting procedure was less than 5 percent, 95 percent of the time. This experimental error could be further reduced, if desired, by incorporating a larger number of cultures in each experimental set.

SUMMARY AND CONCLUSIONS

A procedure has been developed for preparing and handling large numbers of replicate cultures of a pure strain of cells growing in massive cultures on surface substrate such as cellophane in a medium of embryo extract (1:1), horse serum, and saline. The cultures were scraped from

the substrate, passed through 80- and 150-mesh platinum sieving, stirred for 15 minutes to uniform suspension and delivered in aliquots to T–12 flasks. The suspension was equilibrated with a gas mixture of 5 percent carbon dioxide in air. Details for reproducing the equipment to prepare these cultures have been given. The method of, and equipment for, handling fluid renewals on the cultures have been described. The renewals are effected by centrifuging the cultures for 20 minutes at 1,100 r. p. m., removing the fluid with a stabilized pipetting system and replacing with fresh fluid. The cells are then resuspended by vigorous shaking.

Experimental validation with statistical evaluation of the data indicates that stirring cells in suspension is noninjurious as judged by the subsequent growth of cultures and that insignificant numbers of cells are lost from the cultures by the method of fluid renewals. Furthermore the experimental error of the final procedure was less than 5 percent. This procedure for preparing and handling large numbers of replicate cultures used with the procedure for enumerating cell nuclei (*7*) provides a technique for determining quantitatively the changes that can occur in cultures under a variety of experimental conditions.

REFERENCES

(*1*) Sanford, K. K., Earle, W. R., and Likely, G. D.: The growth in vitro of single isolated tissue cells. J. Nat. Cancer Inst. 9: 229–246, 1949.

(*2*) Evans, V. J., and Earle, W. R.: The use of perforated cellophane for the growth of cells in tissue culture. J. Nat. Cancer Inst. 8: 103–119, 1947.

(*3*) Earle, W. R., Evans, V. J., and Schilling, E. L.: Extension of cellophane substrate procedure to growth in vitro cultures of large areas. J. Nat. Cancer Inst. 10: 943–967, 1950.

(*4*) Flask design to be published.

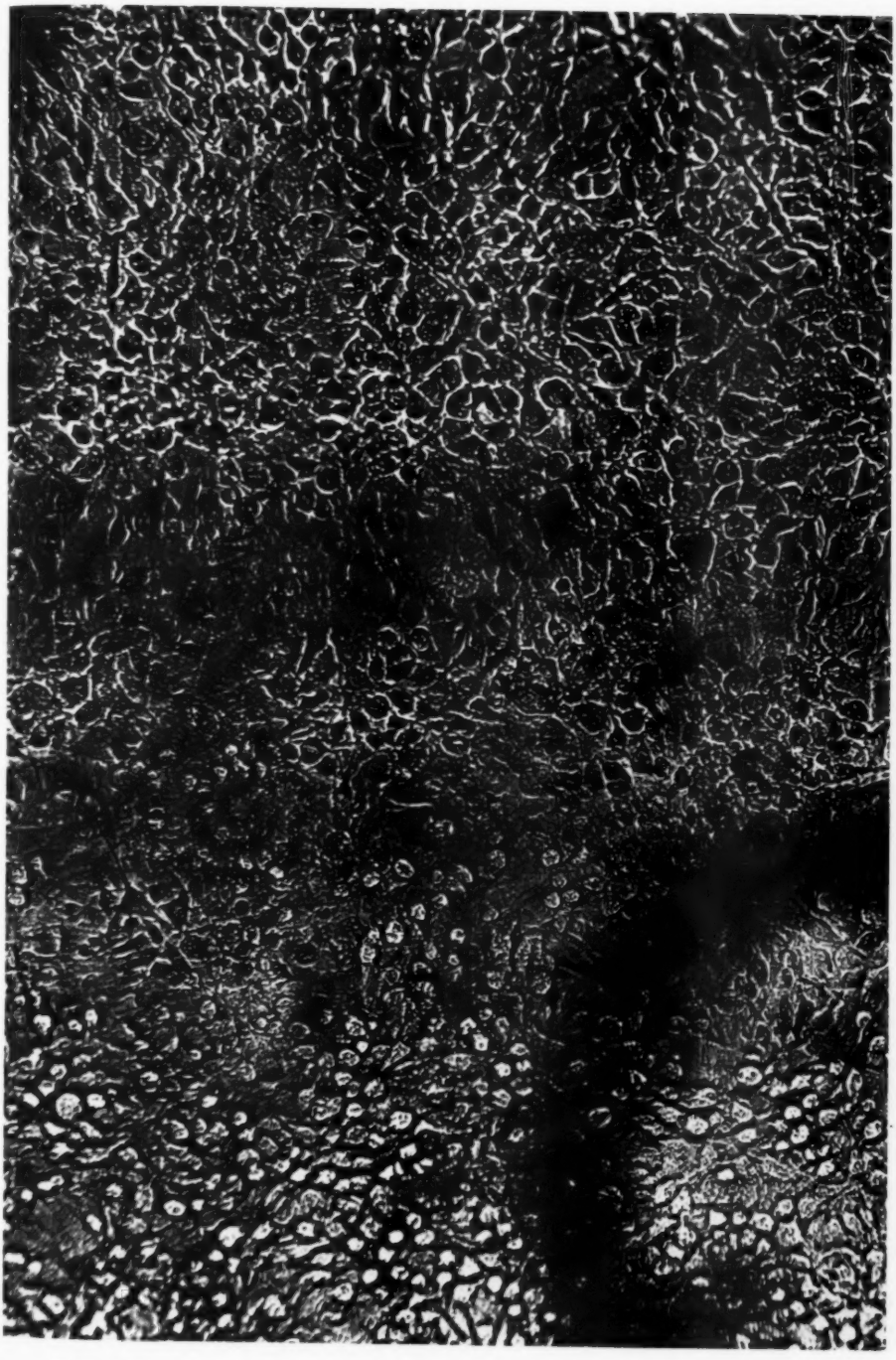
(*5*) Earle, W. R., Evans, V. J., Edward, M. F., and Duchesne, E.: Influence of perforation design on growth of tissue cells under perforated cellophane sheets in vitro. J. Nat. Cancer Inst. 10: 291–295, 1949.

(*6*) Earle, W. R.: Production of malignancy in vitro IV. The mouse fibroblast cultures and changes seen in the living cells. J. Nat. Cancer Inst. 4: 165–212, 1943.

(*7*) Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., and Shannon, J. E.: The measurement of proliferation of tissue cultures by enumeration of cell nuclei. J. Nat. Cancer Inst. 11: 773–795, 1950.

Plate 98

Figure 1.—Strain L mouse cells growing under perforated cellophane. This photograph shows the approximate cell density and condition of cultures used for stocks of cells. Approximately 3,000,000 cells per milliliter of culture fluid are in such a culture. × 200



BIOLOGICAL ACTIVITY OF THE AGENT OF CHICKEN TUMOR I (ROUS) IN CITRATE BUFFERS OF VARIOUS MOLAR CONCENTRATIONS[1]

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The following investigation was part of a general quantitative study that is being made of the factors that influence the stability of the agent of chicken tumor I (Rous sarcoma) (*1*) and of the conditions under which this agent may be handled in the laboratory without appreciable loss of tumor-producing potency.

Previous observations carried out at 37° C. indicated that the biological responses were ideally reproducible when citrate buffers were used as media for suspending the agent; that is, the variations in results from experiment to experiment were no greater than were to be expected from the error of the bioassay method (*2*). The observations involved various levels of pH, from 4.5 to 7.0, but only one concentration of citrate, namely 0.05 M. The present investigation was for the purpose of ascertaining the effect of molarity on tumor-producing activity, and of determining the optimum molarity, or molarity range, of citrate buffers for quantitative biological studies on the agent of chicken tumor I.

MATERIALS AND METHODS

Citrate buffers.—The pH of all citrate buffers was 6.7, which was within the range giving maximal biological activity in a previous investigation (*2*).

Tumor agent.—Aqueous suspensions of partially purified tumor agent were prepared by differential centrifugation of tumor-tissue extracts as previously described in detail (*3*); they represented suspensions of the microsome fraction derived from first saline (0.9 percent NaCl) extracts of frozen tumor tissue by 1 complete cycle of differential centrifugation. In a given experiment, a common lot of aqueous tumor-agent suspension was employed for the further preparation of all buffer-agent mixtures, and of the standard reference suspensions used for establishing the 100-percent potency level in the bioassays.

Agent-buffer mixtures.—In experiments 1–4 the agent-buffer mixtures

[1] Received for publication November 13, 1950.

having a final citrate concentration of 0.5 M or less were prepared by mixing equal volumes of aqueous tumor-agent suspension and citrate buffer of double the strength desired in the final mixture. Because of the limited solubility of sodium citrate, a different procedure was required for the 1.0 M materials. They were prepared with 1.5 M buffer and stronger suspensions of tumor agent in distilled water, which were adjusted so that they gave the final desired concentration of both tumor agent and citrate after mixing. The agent-buffer mixtures employed in experiment 5 were prepared by adding 1 volume of a 32-times-concentrated agent suspension to 31 volumes of saturated [2] buffer. The resulting mixtures were therefore $^{31}/_{32}$, or about 97 percent, saturated with respect to citrate. They were estimated to be about 1.6 to 1.7 M.

Bioassays.—The relative potency of each test mixture of buffer and tumor agent was determined by comparison with a paired sample of a standard reference suspension in a separate bioassay determination involving 36 chickens and 144 inoculation sites. The 4 × 4 Latin-square design was employed, as previously described (*3*, *4*, *5*), for balancing the biological variables. However, 3 different Latin-square patterns were employed in each assay, and there were 3 replicates of each of 3 basic sets (4 chickens and 16 sites) instead of 10 replicates of a single set as in previously reported studies. The errors of the bioassays were determined by the statistical procedures described by Cornfield [see appendix to reference (*2*)].

The agent suspensions used as standards of reference for establishing the 100-percent potency level were prepared in each experiment from the same lot of aqueous tumor-agent suspension as was used for the test mixtures. In all experiments except 1 (number 5) aliquots of the aqueous suspension were mixed with equal volumes of 0.2 M citrate buffer, of pH 6.7, to produce standard mixtures having a citrate concentration of 0.1 M, and an agent concentration equal to that in the test mixtures. In experiment 5 the standard mixtures had a final citrate concentration of 0.05 M.

All agent preparations, both the test and the standard mixtures, were diluted $^1/_{10}$ and $^1/_{100}$ for the purpose of bioassay [see reference (*5*)]. The diluent employed in experiments 1–4 was 0.1 M citrate buffer of pH 6.7. In experiment 5, which involved saturated citrate buffer, the dilutions of the test mixtures were made with distilled water. The standard mixtures of experiment 5 were diluted with 0.05 M citrate buffer for the purpose of bioassay.

Experimental procedures.—Because of the relatively large number of materials to be tested in most of the experiments (*i. e.*, 10 or 12) and the amount of time required for inoculating the test chickens for the assay of a given material (15 to 20 minutes), they were not all prepared at the same time, but individually, at 15- to 20-minute intervals. At previously scheduled times, identical samples of aqueous tumor-agent suspension were taken for the preparation of 1) a test, and 2) a corresponding standard mixture.

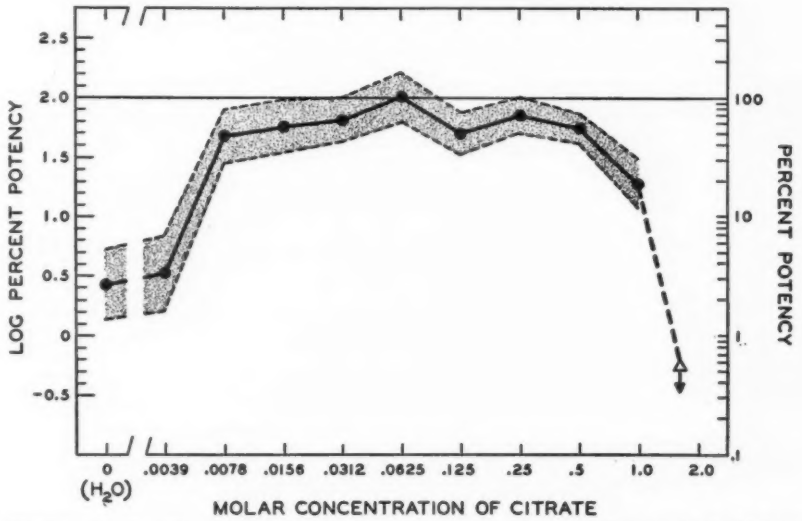
[2] Saturated at room temperature and cooled to 6°–8° C. before used.

As each paired set of test and standard mixtures was prepared the standard was placed in a refrigerator (6°–8° C.) where it was kept until used for the bioassay, and the test mixture was placed in a water bath at a temperature of either 37° C. or 6°–8° C., depending upon the temperature condition being tested. After exactly 3 hours it was removed from the water bath and, if at 37° C., it was chilled by immersion in an ice-water bath.

In all experiments except that with saturated citrate buffer (experiment 5) the molarities of the test mixtures were adjusted to the constant level of 0.1 as the mixtures were diluted for inoculation into test chickens. This was also the molarity used in preparing all of the corresponding standard mixtures. The test mixtures of experiment 5 could not be adjusted to the constant citrate molarity of the previously executed experiments, by simple adjustment procedures, without diluting the tumor agent more than was desired. No attempt was made, therefore, to standardize the molarity of the inoculated materials in this experiment.

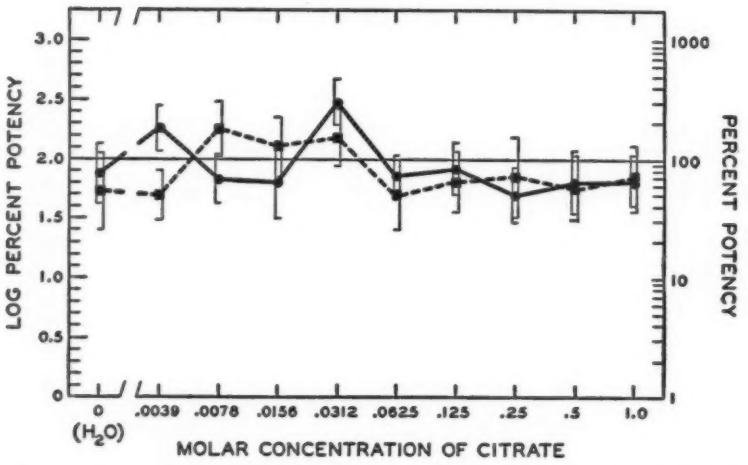
EXPERIMENTS AND RESULTS

The molar concentrations of citrate and the temperature conditions employed in experiments 1–5 are given in text-figures 1–4.



TEXT-FIGURE 1.—Tumor-producing potencies of suspensions of the agent of chicken tumor I in citrate buffers of various molar concentrations after standing for 3 hours at 37° C.

circles—data of experiment 1
triangle—data of experiment 5A
shaded band—error zone limited by ±1 standard-error unit.

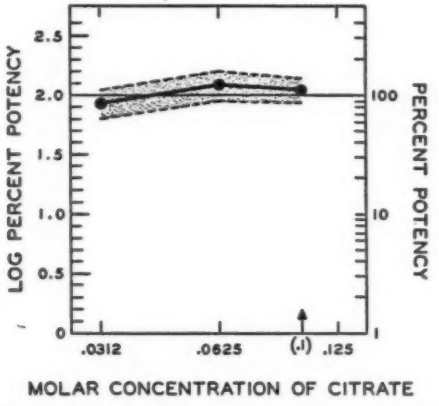


TEXT-FIGURE 2.—Tumor-producing potencies of suspensions of the agent of chicken tumor I in citrate buffers of various molar concentrations after standing for 3 hours at 6°–8° C.

circles—data of experiment 2

squares—data of experiment 3

brackets—error zone limited by ±1 standard-error unit.

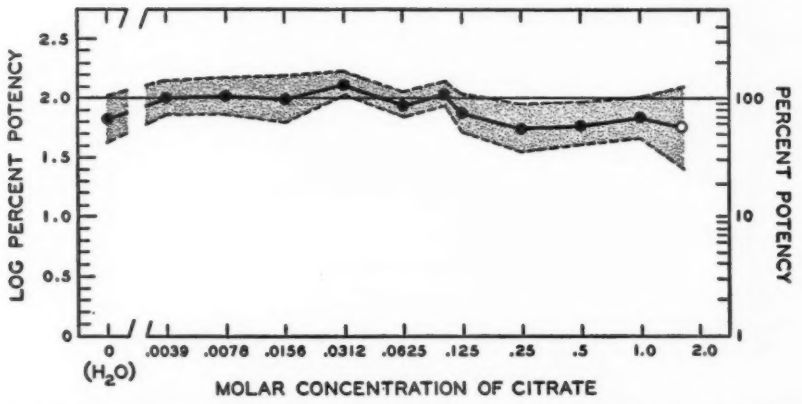


TEXT-FIGURE 3.—Results of quadruplicate bioassays on suspensions of the agent of chicken tumor I after 3 hours at 6°–8° C. Data of experiment 4.

See legend of text-figure 2.

Results at 37° C.

As shown by the plotted points of text-figure 1, there was no statistically significant peak or particular point of optimal citrate concentration for the biological activity of the agent of chicken tumor I after incubation for 3 hours at a temperature of 37° C. There was, however, a broad range of molar concentrations, 0.0078 to 0.5, within which the activity remained



TEXT-FIGURE 4.—Tumor-producing potencies of suspensions of the agent of chicken tumor I in citrate buffers of various molar concentrations after standing for 3 hours at 6°–8° C.

closed circles—average results of experiments 2, 3, and 4.

open circle—result of experiment 5B.

shaded band—error zone limited by ±1 standard-error unit.

The smaller standard errors for the mean potencies at citrate concentrations 0.0312–0.1 M are due to larger numbers of biological observations at these concentrations (see text).

relatively high and was only slightly lower, on an average, than that of the standard reference suspensions. The individual potency estimates within this range did not differ significantly from the potency of the reference standards (100 percent; 2.0 log percent) but all but one of them fell below the standard, and, taken together, they represent a statistically significant decrease in activity at a temperature of 37° C.

Since the standard of reference used for the bioassays was simply an unincubated suspension of tumor agent in 0.1 M citrate buffer, it also represented a true control for the effect of temperature at molarity 0.1. Although there was no test mixture of exactly 0.1 M concentration, a potency value for this molarity can be estimated from the smooth curve which can be drawn through all of the points along the plateau in text-figure 1. Using a smoothed curve which had the slight curvature indicated by the plotted points, the log-percent potency value of 1.85 was estimated for an incubated suspension at molarity 0.1. This corresponds to a potency of about 70 percent as compared with the standard, and indicates that at this molarity the potency of tumor-agent suspensions incubated for 3 hours at 37° C. might be expected to be about 30 percent lower than that of similar suspensions kept at 6°–8° C.

The potency estimates obtained with distilled water, with citrate concentrations 0.0039 M and 1.0 M, and with saturated citrate buffer (about 1.6 to 1.7 M) were significantly lower than the potency of the standard ($P<0.01$ in all instances). It is apparent, therefore, that the biological activity of the incubated tumor-agent suspensions dropped off rapidly as

the concentration of citrate was decreased below 0.0078 M or increased above 0.5 M.

Although there was some loss in biological activity at all citrate concentrations when the materials were incubated at 37° C., the loss at concentrations 0.0078 to 0.5 M was not sufficiently pronounced, within a period of 3 hours, to preclude quantitative investigations on the tumor agent at this temperature. The results of the present and previous (*2*) investigations indicate that when citrate buffers are used, biological observations on the agent of chicken tumor I are highly reproducible after incubation at 37° C.

The results obtained with citrate concentrations lower than 0.0078 M and higher than 0.5 M indicate that the loss in activity is too great for these concentrations to be used in investigations on the agent at this temperature.

Results at 6°—8° C.

Two identical experiments (numbers 2 and 3) were carried out in which agent-buffer mixtures varying in citrate concentration from 0.0039 to 1.0 M in 2-fold steps, were tested after standing for 3 hours at a temperature of 6°–8° C. In the first of these (experiment 2; see circular points of text-figure 2) all but one of the potency estimates, including that for the distilled water control, fell within limits of experimental error of the potency of the reference standard. The exception, namely, that for citrate concentration 0.03125 M, was greater than the reference potency by 2.51 times its standard error, the deviation approaching the 1-percent level of significance. This suggested that a slightly, but possibly significantly higher potency was obtained at concentration 0.03125 M of citrate.

Because this borderline result could not be interpreted conclusively, and because of the importance of possible implications of a true optimal concentration of citrate, the experiment was repeated under identical conditions (experiment 3; see square points of text-figure 2). The potency estimate for citrate concentration 0.03125 M fell above the standard in experiment 3, as in the preceding experiment, but the deviation in this instance was not statistically significant. The result was not inconsistent with that of the preceding experiment, however, since the standard errors of the 2 results overlapped. Their mean (231.4 $\begin{cases}+96.7\\-68.2\end{cases}$ percent; 2.364 $\pm$0.152 log percent) was higher than the standard potency by 2.39 times its standard error, and the question of an optimal concentration of citrate at molarity 0.03125 therefore remained equivocal ($0.01<P<0.05$).

Since the evidence for an optimum rested primarily on a single observation of borderline significance, and, because uncontrolled sources of variation (*i. e.*, sources which are not taken into account by the statistical procedures) may sometimes enter into biological studies, another experiment was performed to investigate the question further using larger numbers of test chickens for the bioassays (experiment 4; see text-figure 3). The experiment was performed under the same general conditions as the other experiments at 6°–8° C., but only 3 concentrations of citrate were

investigated, namely, 0.03125, 0.0625, and 0.1 M, and quadruplicate tests were carried out at each concentration.

As may be seen from text-figure 3 the results were the same, within limits of experimental error, for all 3 concentrations of citrate and in no instance was there a significant deviation from the reference standard. The question of an optimal concentration at molarity 0.03125 was therefore ruled out of practical consideration by the more precise bioassay results of this experiment.

The quadruplicate samples of the 0.1 M citrate concentration in experiment 4 were identical in every respect with the samples of standard with which they were compared in the bioassays. Their potencies were known, therefore, to be the same as the standards, and the estimate of potency derived from the combined bioassay data provides a check on the accuracy of the bioassay method under the particular conditions involved in these experiments. The estimated potency value, namely, $109.3 \begin{cases} +30.2 \\ -23.7 \end{cases}$ percent (2.039 ± 0.106 log percent) was well within limits of error of the standard (100 percent; 2.000 log percent), and the reliability of the assay conditions and procedures was, therefore, confirmed.

The result obtained with saturated citrate buffer at 6°–8° C. (experiment 5) is shown by the open circle at the extreme right of text-figure 4. Also included in this figure are the averages of all of the results of the preceding experiments carried out at 6°–8° C. (the smaller standard errors shown for the averages at citrate concentrations 0.03125 through 0.1 M are due to the larger numbers of biological observations at these levels). It is seen that the potencies were the highest, and were essentially the same in the tumor-agent suspensions having concentrations of citrate of from 0.0039 through 0.1 M. The average potency within the molarity range of from 0.125 M through essentially complete (97 percent) saturation was 1.81 log percent, or about 65 percent as compared with the reference standard (*i. e.*, the 0.1 M concentration of citrate). Although the potency estimates at these higher concentrations did not differ significantly from the standards as individuals, it is improbable that all 9 of the estimates made in 3 experiments (numbers 2, 3, and 5) would be lower than their corresponding standards due to chance variation alone. Taken together, they represent a relatively slight, but real, decrease in potency at the higher citrate concentrations. The average decrease of about 35 percent was not sufficient to preclude use of the stronger citrate concentrations in quantitative investigations on the agent at 6°–8° C. The fact that the potencies did not show a regression on concentration, but rather indicated a similar slight reduction at all levels above 0.125 M, suggests that the effect may have been due to aggregation of the agent particles (or their admixture with other aggregated or insoluble constituents) at the higher citrate concentrations.

The similar, slightly lowered, average potency for the 2 distilled-water suspensions of tumor agent could have resulted due to chance alone. The

biological observations on this type of material were not sufficient for the detection of a real difference in potency amounting to only 39 percent.

DISCUSSION

The results of this study provide quantitative information on the amount of biological activity remaining in citrate buffers of various molar concentrations after a period of 3 hours at each of 2 temperatures (37° and 6°–8° C.). They also confirm the reproducibility of results obtained with citrate buffers (*2*) and provide a basis for the planning of further quantitative investigations on the agent of chicken tumor I under stable experimental conditions.

It should be emphasized that the quantitative potency determinations presented herein represent determinations of tumor-producing potentiality, as indicated by the particular biological response employed [*i. e.*, latent period, see reference (*5*)], and not necessarily the actual total amount of active-agent substance. For example, it is possible that physical aggregation of fully active agent particles in a suspension might lower the tumor-producing potentiality of the suspension although the total amount of active agent remained unchanged. For this reason it is not possible to interpret definitely the significance of slight (*i. e.*, in relation to the bioassay error) changes in potency such as those observed with the higher concentrations of citrate at 6°–8° C. The highly significant (*i. e.*, in comparison with the bioassay error) decreases in tumor-producing potentiality observed with some concentrations of citrate at 37° C., on the other hand, could not be entirely explained by the phenomenon of aggregation, and they therefore undoubtedly represent some considerable loss in the actual total amount of active-agent substance.

The fact that concentrations of citrate up to that of practically complete saturation were tolerated for 3 hours at a temperature of 6°–8° C. without appreciable loss in activity, suggests that strong citrate concentrations might be used in conjunction with physical or chemical procedures for the further fractionation of preparations containing the agent such as the microsome suspensions employed in the present experiments. For example, the densities of suspending solutions might be increased, up to about 1.25, as an aid to separation of the microsome constituents by differential centrifugation; or, possibly, strong concentrations of citrate might be used for their chemical separation in a manner similar to that in which strong concentrations of ammonium sulphate have been used, but with less damaging effects on the highly labile tumor agent.

SUMMARY

Quantitative determinations were made of the biological activity of suspensions of the agent of chicken tumor I (Rous) in pH 6.7 citrate buffers of various molar concentrations. Observations were carried out both at temperatures of 37° C. and of 6°–8° C.

At 37° C. there was some loss in potency during a period of 3 hours at all concentrations of citrate tested; but, within the concentration range

0.0078 to 0.5 M, the loss averaged only about 30 percent, and was therefore not sufficient to preclude the use of citrate buffers for investigations on the agent at this temperature.

The agent remained fully active at 6°–8° C. in all concentrations of citrate from 0.0039 to 0.1 M. Higher concentrations, up to practically complete (97 percent) saturation, were tolerated at this temperature without excessive loss (*i. e.*, only about 35 percent, on an average) in tumor-producing activity.

REFERENCES

(*1*) Rous, P.: A sarcoma of the fowl transmissible by an agent separable from the tumor cells. J. Exper. Med. 13: 397–411, 1911.

(*2*) Bryan, W. R., Maver, M. E., Moloney, J. B., Sells, M. T., and White, C. L.: Comparative stability of the agent of chicken tumor I in citrate and phosphate buffers at 37° C. J. Nat. Cancer Inst. 11: 269–277, 1950.

(*3*) Bryan, W. R., Maver, M. E., Moloney, J. B., Thomas, M. A., and Sells, M. T.: Comparative stability of the agent of chicken tumor I in phosphate and borate buffers during incubation for 3 hours at 37° C. J. Nat. Cancer Inst. 10: 647–656, 1949.

(*4*) Bliss, C. I., and Marks, H. P.: The biological assay of insulin. II. The estimation of drug potency from a graded response. Quart. J. Pharm. & Pharmacol. 12: 182–205, 1939.

(*5*) Bryan, W. R., Riley, V. T., Deihl, D. G., and Voorhees, V.: Studies on the agent of chicken tumor I. I. The relative potencies of serial extracts and their comparative values as source materials for purification studies. J. Nat. Cancer Inst. 7: 447–453, 1947.

NUCLEOLAR VOLUME CHANGES IN THE MOUSE PANCREAS AFTER REPEATED PILOCARPINE INJECTIONS[1,2]

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Evidence from ultraviolet cytochemical studies on different types of cells indicates an important role of the nucleolus and the nucleolus-associated chromatin in the formation of cytoplasmic protein (*2–5*, *19*, *20*). Although nucleolar enlargement is found under various conditions, its frequent occurrence in actively growing and protein-producing gland cells, both of which contain increased amounts of ribose nucleotides and protein in the cytoplasm, merits further investigation.

Caspersson, Landström-Hydén, and Aquilonius (*5*) have reported that after pilocarpine injections the cytoplasmic nucleotides and protein are considerably reduced in the pancreatic acinar cells but are rapidly resynthesized during the restoration of zymogen granules. At this time the nuclear membrane also shows a stronger ultraviolet absorption and is surrounded by large cytoplasmic masses of nucleotides. The present study was undertaken to obtain data on the relationship of nucleolar volume changes to protein production in the pancreatic acinar cells. It was found that there is a definite increase in the nucleolar volume during the resynthesis of zymogen granules after their discharge following pilocarpine stimulation.

MATERIALS AND METHODS

Strain A male mice, 10 to 13 weeks old, were used. Several preliminary experiments were performed to establish suitable dosages. Nineteen animals each weighing 24 ± 1 gm. were selected and fasted for 18 hours prior to injections. After fasting the average weight decreased to 21 ± 1 gm. The animals were kept in individual wire-mesh cages and fasted throughout the experiment. Each mouse was given 0.1 cc. per 10 gm. of body weight of a 0.5 percent solution of pilocarpine hydrochloride (NF) intraperitoneally. This dosage was given 6 times at hourly intervals. Twelve control animals of the same age and weight were subjected to the same experimental conditions except that they received comparable injections of distilled water.

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[2] Aided by grants from the National Cancer Institute and the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

The animals were killed at ½, 6, 14, 24, and 48 hours after the last injection. The tail of the pancreas was fixed in Maximow's modification of Helly's fluid, using neutralized formalin, and paraffin sections were cut at 4 microns. The procedure for the preparation of tissues was standardized as much as possible in order to minimize variations in results due to technique. Some slides were stained by Feulgen's technique and counterstained with alcoholic fast green FCF; others were stained with hematoxylin-eosin and with eosin-azure II.

Nucleolar diameters were measured with a filar micrometer eyepiece from Feulgen preparations with the use of a green filter. All measurements were made by one of the authors, since experience in our laboratory has shown that several observers who may obtain slightly different averages from the same sample are consistent within themselves. The nucleoli were measured from groups of adjacent nuclei in different areas which were selected to give adequate representative sampling. Nucleoli in at least 50 nuclear sections were measured for each animal, with the total number of nucleoli exceeding 100 in all cases. All the nucleoli for each nuclear section were recorded in order to obtain the average total nucleolar volume per nucleus. Diameters were measured for the nucleolar material only, excluding the nucleolar-associated chromatin (heterochromatin) stained by the Feulgen reaction. For computation it was assumed that the nucleoli were spherical, and the volumes were calculated from the formula $4/3\ \pi\ r^3$. Errors resulting from slight irregularities in the shape of nucleoli are obviated by the large numbers of these measurements for each tissue. Although the results are presented in cubic microns, the values serve for comparative purposes only, since differences in the method of fixation and subsequent treatment affect the volumes of different components of tissues (*18*). The data were analyzed statistically to determine the significance of the results.

A separate series of 30 male animals was divided into 5 groups of 6 each. One group served as normal controls and the others were fasted for 18, 36, 48, and 72 hours in order to observe the histologic appearance of the acinar cells during prolonged fasting.

RESULTS

The effects of pilocarpine were manifest within 10 minutes after each injection by copious weeping, diarrhea, and salivation. The pancreas appeared whiter and firmer than in the controls at the first half hour after the last injection.

After the pilocarpine treatment the pancreatic acinar cells showed diminished amounts of zymogen granules. Although there was some variation in cell size in different animals, there appeared to be a slight tendency toward enlargement of the cells when compared with those of control animals. In most instances the majority of the cells were enlarged to the same degree in one pancreas. Some animals, however, showed no noticeable increase in cell size over that of controls, and this may have been due to individual variations at the beginning of the experiment or

differences in response. Variations in morphological and chemical composition of the exocrine pancreas after fasting have been observed by others (*10*, *21*). By 24 hours after the last injection the granules were fully restored in the acinar cells regardless of cell size. Throughout the experiment the control animals possessed small- to moderate-sized cells with variable amounts of stored granules. Large cells were present only around islets and blood vessels.

Various degrees of cytoplasmic vacuolation occurred in the acinar cells at ½, 6, and 14 hours after the pilocarpine injections. Many small vacuoles were present in the intermediate zone of the cytoplasm between the basal and granular portions, particularly in enlarged cells. These vacuoles sometimes appeared as clear spaces around single or confluent zymogen granules. When large vacuoles were present, they were localized chiefly in the basal portion of the cytoplasm. The cells from the 24- and 48-hour groups contained few if any vacuoles. The control animals showed only a few vacuoles in an entire section or none at all.

A definite increase in nucleolar volume was observed in the acinar cells during the recovery period after repeated pilocarpine injections. The results are presented in table 1. Statistically significant enlargement was

TABLE 1.—*Nucleolar volumes of pancreatic acinar cells*

No. of hours after last injection	No. of mice	No. of nucleoli measured	No. of nuclei	Average nucleolar volume in μ^3	Average nucleolar volume in μ^3 per nucleus
Control Mice					
½–6	4	508	297	2. 19	3. 75
14	3	300	184	2. 31	3. 76
24–48	3	323	178	2. 39	4. 33
Experimental Mice					
½	3	307	189	2. 97	4. 82
6	4	401	246	3. 00	4. 89
14	3	305	183	3. 20	5. 34
24	4	415	233	2. 30	4. 10
48	4	401	270	1. 83	2. 72

found in the ½-, 6-, and 14-hour groups when compared with their respective control animals. The average nucleolar volume as well as the average total nucleolar volume per nuclear section returned to normal at 24 hours and was below normal at 48 hours.

Figure 2 illustrates the acinar cells with large nucleoli from an animal that was sacrificed 14 hours after the last injection of pilocarpine. These cells had an average nucleolar volume of 3.21 cubic microns and an average total nucleolar volume of 5.26 cubic microns per nuclear section. The respective volumes from a control tissue (fig. 1) for the same period were 2.19 and 3.77 cubic microns.

Intranucleolar structures, which appear to be vacuoles similar to those described by Saguchi (*17*) for frog pancreas, were observed in the control

and experimental tissues. They appeared colorless with all three staining methods employed. When multiple vacuoles were present, they imparted an alveolar appearance to the nucleolus. The number of nucleoli containing these intranucleolar structures was more numerous in some of the experimental animals than in the control animals.

Fasting alone caused storage of the granules and a slight to moderate enlargement of cells at 18 hours. This increase in size, however, was neither as great nor as uniform as after pilocarpine treatment. Those animals fasted for 36, 48, and 72 hours showed small to moderate-sized cells, with varying degrees of granule storage.

DISCUSSION

The pancreatic acinar cells show a significant nucleolar enlargement during the rapid synthesis of the protein-rich zymogen granules after intense stimulation. This result is in accord with the current concept of nucleolar change under different growth conditions and its active function in the production of large amounts of cytoplasmic protein. Of the various samples of tissues removed at different intervals after stimulation, those collected at 14 hours exhibited the largest average nucleolar volume, after which there was a decrease in size. The volumes approached the control values at 24 hours, by which time the restoration of zymogen granules had apparently been completed in the majority of the cells. Van Weel and Engel (*21*) showed chemically that, following an intravenous injection of pilocarpine, the carboxypolypeptidase content of the mouse pancreas dropped sharply at 3 hours and returned to normal levels at 12 hours, with closely related changes in the amount of zymogen granules. Apparently the time required for the cells to reconstitute their granules depends upon the intensity and duration of the stimulus (*9*, *15*).

Caspersson and co-workers (*2*, *3*) suggest that the nucleolus-associated chromatin produces the protein substances that accumulate in the nucleolus from which there is a migration toward the nuclear membrane. Ribose nucleic acids are then produced in the juxtanuclear cytoplasm with a simultaneous augmentation of cytoplasmic protein. Subsequent to pilocarpine treatment the pancreatic cells contain increased concentrations of nucleic acid in the basal portions of the cytoplasm, especially near the nuclear membrane (*5*, *15*). It is believed that the nucleolus plays a significant role in this system and that its enlargement precedes the increase in ribose nucleic acid and protein in the cytoplasm. Ludford, Smiles, and Welch (*13*) disagreed somewhat with the above view. They observed in ultraviolet micrographs from living malignant cells that the cytoplasmic absorption occurs in the more peripheral parts of the cells and not in the vicinity of the nucleus. They suggested that the mitochondrial-cytoplasmic interfaces might be the site of protein synthesis.

The nucleolar volume evidently changes under different conditions of growth and function (*2*, *8*, *19*). Stowell (*19*), studying the regenerating rat liver after partial hepatectomy, found an enormous increase in the nucleolar volume, the peak of which occurred at 24 hours. Ultraviolet

absorption measurements for comparable materials showed that a significant increase in the nucleic acid content of the nucleoli followed at 36 hours of regeneration and was still higher at 42 hours. Although some investigators have stressed the significance of large nucleoli in malignancy (*14*), later work (*7*, *20*) has shown that this cannot be used as the sole criterion for malignancy.

In the present study no attempt was made to determine the nature of the intranucleolar vacuoles. Page, Regan, and MacCarty (*16*) observed the effects of different techniques of fixation and staining on these structures in normal and neoplastic human tissues. Despite the lack of direct evidence, they believed as did Saguchi (*17*) that the argentophile bodies after formalin fixation were identical with the vacuoles after other fixatives. These investigators found that the more malignant tissues contained greater numbers of these intranucleolar vacuoles. Lewis (*12*) observed these structures in some but not all malignant fibroblasts. Brock, Stowell, and Couch (*1*) described their occurrence in mouse liver.

Cytoplasmic vacuolation of the pancreatic acinar cells may be produced by pilocarpine (*6*) and other parasympathetic stimulation. Leblond and Sergeyeva (*11*) observed this after treatment with a potent parasympathetic drug, acetylcholine. They could not demonstrate fatty substances with Sudan III and concluded that vacuolation was due to autodigestion from activation of zymogen granules.

Although no measurements of the acinar cells were made, a slight tendency toward enlargement of these cells was observed after pilocarpine injections. This may possibly be due to a compensatory response after repeated intense stimulation. Such results were obtained in the preliminary studies as well as in the final experiment reported here.

SUMMARY

Repeated intraperitoneal injections of pilocarpine cause an enlargement of the nucleoli in the pancreatic acinar cells of mice. This occurs concomitantly with the restoration of zymogen granules and would tend to support the current view that the nucleoli actively participate in the metabolism of cells engaged in production of large quantities of cytoplasmic protein.

REFERENCES

(*1*) Brock, B., Stowell, R. E., and Couch, K.: Staining of intranuclear lipids of mouse liver under different conditions of growth. In press.

(*2*) Caspersson, T. O.: Cell growth and cell function. A cytochemical study. W. W. Norton & Co., New York, 1950.

(*3*) Caspersson, T., and Schultz, J.: Ribonucleic acids in both nucleus and cytoplasm, and the function of the nucleolus. Proc. Nat. Acad. Sc. 26: 507–515, 1940.

(*4*) Caspersson, T., and Santesson, L.: Studies on protein metabolism in the cells of epithelial tumours. Acta Radiol., Suppl. 46: 1–105, 1942.

(*5*) Caspersson, T., Landström-Hydén, H, and Aquilonius, L.: Cytoplasmanukleotide in eiweissproduzierenden Drüsenzellen. Chromosoma 2: 111–131, 1941.

(6) COVELL, W. P.: A microscopic study of pancreatic secretion in the living animal. Anat. Rec. 40: 213–224, 1928.

(7) COWDRY, E. V., and PALETTA, F. X.: Changes in cellular, nuclear, and nucleolar sizes during methylcholanthrene epidermal carcinogenesis. J. Nat. Cancer Inst. 1: 745–759, 1941.

(8) GATES, R. R.: Nucleoli and related nuclear structures. Bot. Rev. 8: 337–409, 1942.

(9) HIRSCH, G. C.: Die Lebendbeobachtung der Restitution des Sekretes im Pankreas. 1 Teil. Die Restitution der Granula nach Pilokarpinreizung, ihr Ort und ihre Zeit. Ztschr. f. Zellforsch. u. mikr. Anat. 15: 36–68, 1932.

(10) HIRSCH, G. C.: Die Lebendbeobachtung der Restitution im Pankreas. IV. Teil. Die Restitution der Drüse als ganzes nach Pilokarpinreizung. Mit einem Exkurs über synchrone, hemisynchrone und asynchrone Zellarbeit. Ztschr. f. Zellforsch. u. mikr. Anat. 15: 290–310, 1932.

(11) LEBLOND, C. P., and SERGEYEVA, M. A.: Vacuolation of the acinar cells in the pancreas of the rat after treatment with thyroxine or acetylcholine. Anat. Rec. 90: 235–242, 1944.

(12) LEWIS, W. H.: Nucleolar vacuoles in living normal and malignant fibroblasts. Cancer Research 3: 531–536, 1943.

(13) LUDFORD, R. J., SMILES, J., and WELCH, F. V.: The study of living malignant cells by phase-contrast and ultra-violet microscopy. J. Roy. Micr. Soc. 68: 1–9, 1948.

(14) MACCARTY, W. C.: The value of the macronucleolus in the cancer problem. Am. J. Cancer 26: 529–532, 1936.

(15) NOBACK, C. R., and MONTAGNA, W.: Histochemical studies of the basophilia, lipase and phosphatases in the mammalian pancreas and salivary glands. Am. J. Anat. 81: 343–368, 1947.

(16) PAGE, R. C., REGAN, J. F., and MACCARTY, W. C.: Intranucleolar bodies in normal and neoplastic human tissue. Am. J. Cancer 32: 383–394, 1938.

(17) SAGUCHI, S.: Studies on the glandular cells of the frog's pancreas. Am. J. Anat. 26: 347–422, 1920.

(18) STOWELL, R. E.: Effect on tissue volume of various methods of fixation, dehydration, and embedding. Stain Technol. 16: 67–83, 1941.

(19) ———: Nucleic acids and cytologic changes in regenerating rat liver. Arch. Path. 46: 164–178, 1948.

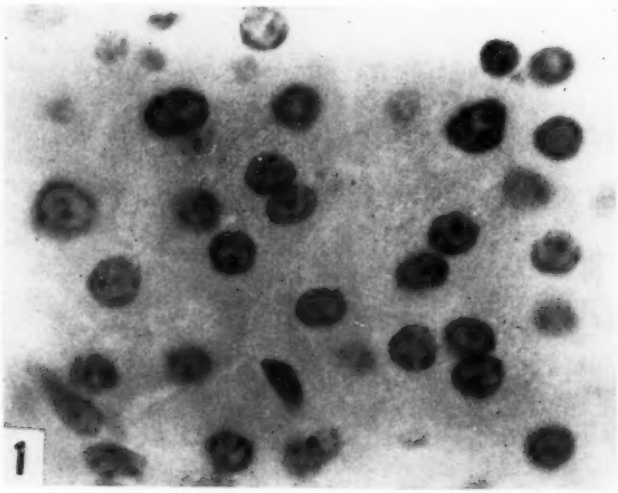
(20) ———: Alterations in nucleic acids during hepatoma formation in rats fed p-dimethylaminoazobenzene. Cancer 2: 121–131, 1949.

(21) VAN WEEL, P. B., and ENGEL, C.: Die Veränderungen des Carboxypolypeptidase-Gehalts vom Pankreas der weissen Maus während der Restitution des Sekretes. Ztschr. f. vgleich. Physiol. 26: 67–73, 1938.

PLATE 99

FIGURE 1.—Acinar cells of the pancreas from a control mouse killed at 14 hours after the last injection of distilled water. Feulgen reaction. × 1,350.

FIGURE 2.—Large nucleoli in the acinar cells of the pancreas from an experimental mouse at 14 hours after the last injection of pilocarpine. Feulgen reaction. × 1,350.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MAMMARY-TUMOR INCIDENCE IN FEMALE C3Hb MICE FOLLOWING LONG-CONTINUED GAMMA IRRADIATION[1]

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INTRODUCTION

Mice of certain strains such as C57 black or L (C57 leaden), assumed not to carry the milk agent, have a very low incidence of mammary tumors (less than 1 percent). On the other hand, mice such as those of strain C3H, which carry the milk agent, have a high-tumor incidence that may reach 95 to 100 percent. It is also well established that hormonal stimulation caused by breeding will increase the mammary-tumor incidence when this incidence is low in virgins carrying the milk agent, such as those of strain A.

This report deals with the effects of irradiation on the mammary-tumor incidence in agent-free C3Hb mice (*1*) derived from a litter taken from the C3H mother by Caesarean section and foster nursing a C57 black female that did not have the milk agent.

Furth and Furth (*2*) and Furth and Butterworth (*3*) were, to our knowledge, the first to study the effect of acute exposures of mice to whole-body roentgen radiation on mammary-tumor development. They found that the over-all incidence of mammary tumors decreased in comparison with that of the controls but the incidence of this tumor was high in those irradiated mice that developed granulosa-cell tumors of the ovaries. They attribute these findings to the fact that following irradiation the ovaries are sterile but continue to produce hormones though in greatly diminished amounts, hence a decreased mammary-tumor incidence during that time. However, later when ovarian tumors of the granulosa-cell type have become established, the incidence increases again as these ovarian tumors produce hormones. Lorenz and Heston (*4*) exposed C3H mice to doses of gamma radiation of 4.4 r, 1.1 r, 0.11 r, and 0.044 r daily (24 hours) for 10 to 15 months to total accumulated doses of 1,000 r and higher at the 4.4 r exposure. Although the females became sterile after having received a total dose of 465 r, and although one group was irradiated with 4.4 r from the time of the fertilization of the ova, the data on mammary-tumor inci-

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dence seemed to indicate that irradiation under the conditions of the experiment had no influence upon the incidence of mammary tumors.

In another series of experiments, Lorenz *et al.* (*5*) irradiated LAF_1 mice beginning at the age of 2 to 3 months with doses of gamma radiation of 8.8 r, 4.4 r, 2.2 r, 1.1 r, and 0.11 r daily (8 hours) for their entire life span. LAF_1 mice are derived from crossing of strain L mothers with strain A fathers; they do not carry the milk agent as was evidenced by a zero incidence in control animals. All experimental groups of females with the exception of the group exposed to 0.11 r showed an incidence of mammary carcinomas ranging from 4 to 14 percent for different dose levels (table 1). Moreover, all experimental groups with the exception of the 8.8 r group irradiated over the entire life span developed sarcomas at the site of the mammary gland in percentages ranging from 13 to 25 percent. They will be referred to subsequently as mammary sarcomas for brevity.

TABLE 1.—*Incidence of mammary tumors in chronically irradiated LAF_1 mice*

Group	Number of animals	Incidence	
		Mammary carcinoma percent	Mammary sarcoma percent
Controls	29	0	0
0.11 r	20	0	25
1.1 r	24	4	25
2.2 r	20	15	25
4.4 r	24	8	21
8.8 r	23	4	0
8.8 r [1]	15	7	13

[1] Limited exposure to 320 r or 680 r, respectively.

No test for the presence of a milk agent was made in this experiment. For this reason, it could not be decided whether or not prolonged irradiation with the small daily doses of gamma radiation might have activated a weak milk agent that was present or destroyed an inhibitor, or whether or not other factors caused the appearance of the tumors. Furthermore, while a few mammary carcinomas and sarcomas were observed in the presence of nonsecreting ovarian tumors, the great majority of the mammary tumors was associated with a granulosa-cell tumor of the ovary. Since no serial sections of the ovarian tumors were made, the possibility existed that the nonsecreting tumors were actually mixed tumors containing granulosa-cell elements.

As it seemed possible, though unlikely, that the induction of mammary carcinomas, and especially of mammary sarcomas, might be a peculiarity of the LAF_1 mice used in these experiments, it was decided therefore to repeat these experiments with a larger number of mice to be exposed to the highest available dose rate of 8.8 r daily (8 hours) only; and to use a different strain of mice, C3Hb, which are free from the milk agent. The history of this strain has recently been given by Heston *et al.* (*1*).

MATERIALS AND METHODS

Ninety-six female strain C3Hb mice were used as experimental animals and 16 virgin females served as controls, mainly for weight measurements and study of the picture of the peripheral blood. A larger number of controls was not necessary as at the same time a group of 160 virgin controls, foster nursed on C57 black mothers, and a group of 188 breeder controls were under study by Heston (*1*). Exposure was started at the age of 1 month and continued until the animals died or were killed when in moribund condition. The experimental arrangement has been given elsewhere in detail (*6*).

The source of the gamma radiation was radium sulfate contained in a capsule of platinum 40×6.4 mm.; 0.5-mm.-wall thickness. In equilibrium, it has at present 990.2 mg. of radium element. The geometric center of each box of experimental animals was placed at approximately 80 cm. from the source. At this distance the gamma-ray intensity at the midpoint of the box was determined with 0.25 r and 25 r ionization chambers to be 1.1 r per hour. Because of the varying distance of the different points inside the box from the source, the gamma-ray intensity varied from point to point, the maximum variations being approximately ±10 percent. Since the mice were forced to feed and drink along the midline of the cages, the variations in total doses among the different experimental mice was probably less than ±10 percent. The daily exposure time was 8 hours.

Blood counts were taken on 16 experimental animals and on the 16 controls. Counts were taken on the experimental animals at the beginning of the experiment at 4-week intervals for 9 weeks, and at 2-week intervals thereafter. Counts on the controls were made similarly, except that the 2-week intervals were not started until the 17th week. The procedure of taking blood samples and the counting technique have been reported elsewhere (*5*). All experimental animals and the 16 control animals were weighed every week. The experiment was started in June 1946 and terminated in April 1948. All surviving animals of the control group of 16 animals were killed when the last experimental animal came to autopsy.

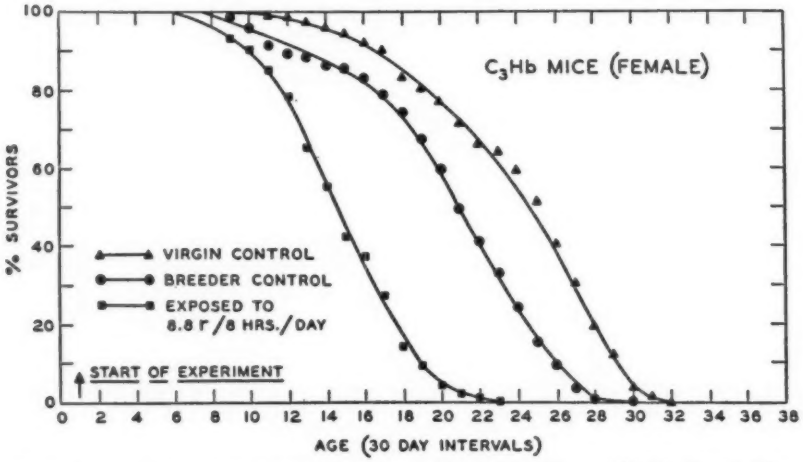
RESULTS

The mean-survival time (zero day=first day of exposure) for the experimental animals was 405±10 days; for the virgin controls (nursed on strain C57 black females), 684±11 days, and for the breeder controls, 576±11 days. The differences in mean-survival time are highly significant. Survival curves are given in text-figure 1, in which the points represent the number of survivors at the end of an interval of 30 days. The mean accumulated dose of the exposed animals was 3,570 r, and the highest accumulated dose reached 5,540 r.

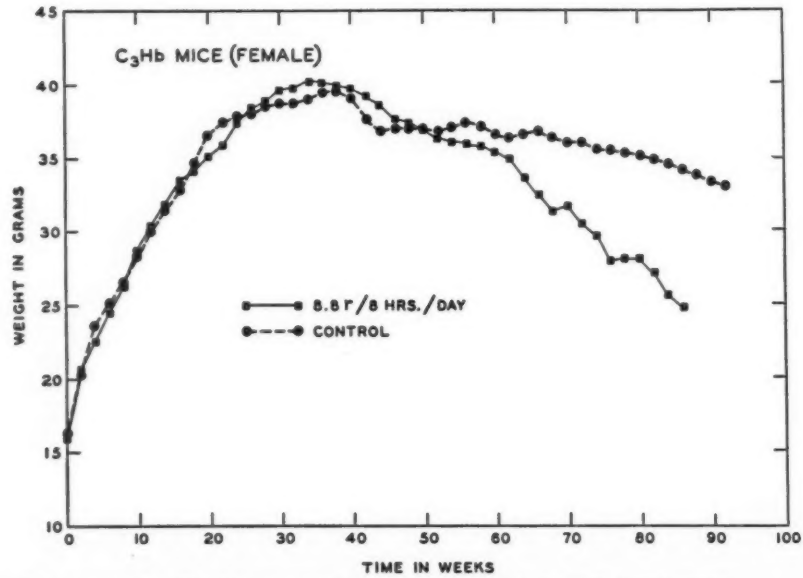
Figure 2 gives the weight curves of the two groups as running averages. Up to approximately 1 year, the weight curves of both groups are the same. Later, the weight of the experimental animals drops more rapidly than

that of the controls, and toward the end the weight of the experimental animals is approximately 70 percent of that of the controls. A similar effect was observed in LAF_1 and strain A mice exposed to 8.8 r daily (8 hours) which showed weights comparable to those of controls for approximately 1-year exposure (*5*).

In plotting the data on the cell content of the peripheral blood generally, all terminal counts of animals in which a drastic reduction of one

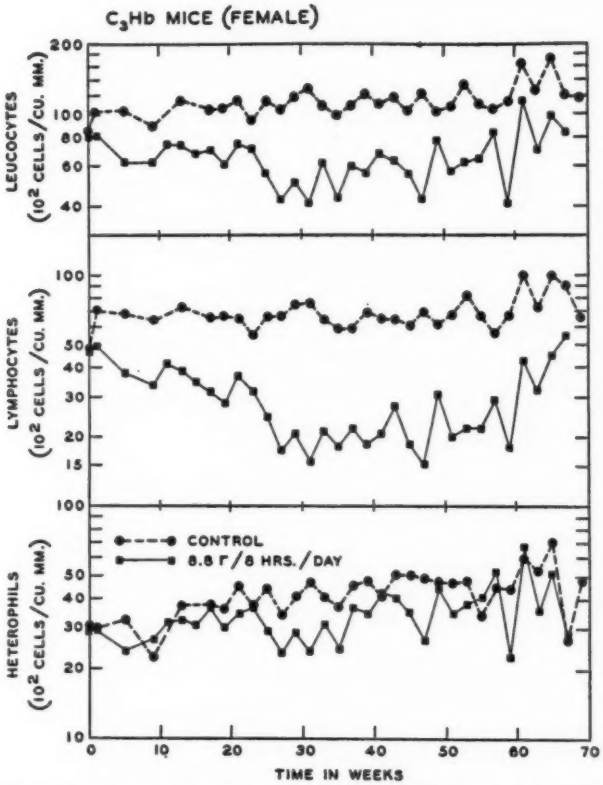


TEXT-FIGURE 1.—Percentage survival of control and experimental C3Hb female mice.



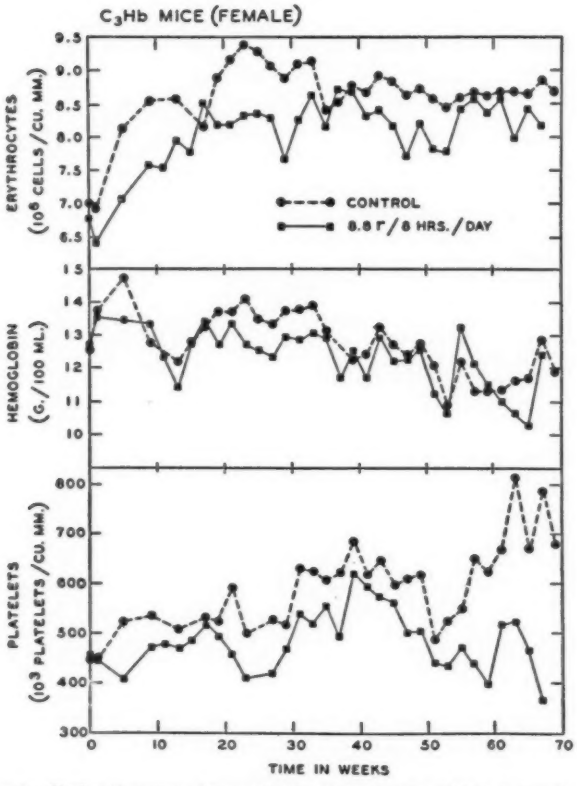
TEXT-FIGURE 2.—Weight curves of irradiated and control C3Hb female mice.

or more of the hematologic constituents indicated imminent death are omitted. Likewise, only the last apparently normal blood count of animals that developed evidence of leukemia in the peripheral blood is included. Text-figure 3 gives the curves for the white-cell counts of the peripheral blood, and text-figure 4 the curves for the red-cell count, hemoglobin content, and platelet count.



TEXT-FIGURE 3.—Leukocyte, lymphocyte, and heterophil counts of controls and of exposed C3Hb mice.

Considering that total doses at the 8.8 r exposure ran up to above 5,000 r, the hematologic constituents of the peripheral blood of the C3Hb mice showed surprisingly little change, and the pattern observed is in complete agreement with that observed in previous experiments with LAF_1 and strain A mice exposed under the same conditions (*5*). Shortly after the exposure started, the leukocyte count began to drop but leveled off after several weeks in comparison with nonirradiated controls. Toward the end of the experiment, control as well as experimental counts rose, the experimental count more than that of the control. This may have been due to an infection of unknown origin in the older animals, but it indicated



TEXT-FIGURE 4.—Red-cell count, hemoglobin content, and platelet count of controls and of exposed C3Hb mice.

that the hematopoietic system of the experimental animals still had sufficient reserve to respond with an increase in leukocytes. The lowered value of the leukocytes in the experimental animals is mainly accounted for by a reduction of the number of circulating lymphocytes, as the curve of the heterophils is only significantly different from the curve of the heterophils of the controls during the 25th to 35th weeks. The depression in the red-cell count of the experimental animals is slight, the hemoglobin content remaining more or less unchanged. The values for the platelets show a more or less constant depression which increases, however, in the last surviving animal.

Ninety-one of the original 96 experimental animals were available for histologic studies. In the remaining 5 animals, post-mortem autolysis was so considerable that histologic studies were not feasible. In tables 2, 3, and 4 the incidence of the most frequent tumors—mammary tumors, lymphoid tumors, and ovarian tumors—are given.

Histologic sections were prepared of the subcutaneous tumors, ovaries, and other neoplasms such as lymphoid tumors, pulmonary tumors, and

hepatomas. The tissues were fixed in Zenker-formol solution (90 parts Zenker's solution and 10 parts formaldehyde), blocked in paraffin, cut, and stained with hematoxylin and eosin. In addition, Wilder's reticulum stain was used on some mammary tumors.

The mammary tumors were examined histologically and sorted according to the criteria outlined by Dunn (*7*). She defined criteria which permitted the classification of mammary tumors into four groups: adenocarcinoma, adenoacanthoma, carcinosarcoma, and sarcoma. These criteria were carefully outlined for mice of the dba strain which were painted with methylcholanthrene. Subsequently Dunn (*1*) has found this classification to be equally applicable to C3Hb breeders. Most of the tumors observed in the C3Hb breeders were adenocarcinomas and adenoacanthomas but one carcinosarcoma and one sarcoma of the mammary-gland region also were found. All four types of mammary tumors induced in dba mice by methylcholanthrene painting were found also in the chronically irradiated C3Hb mice. The applicability of the classification originally devised for the dba mammary tumors to the C3Hb mammary tumors induced by irradiation is permissible by virtue of their similarity and is desirable in facilitating comparison with data from other experiments. Some representative photomicrographs are given in figures 1–6.

The occurrence of the mammary tumors is recorded in table 2. For the compilation of the table, 1 week prior to the first observation of the tumor was taken as the time of appearance of the tumor. The total tumor incidence was 47 percent, with a mean tumor age of 16.2 months. Broken down into mammary carcinomas (adenocarcinoma and adenoacanthoma) and sarcomas, an incidence of 20 percent, with a mean tumor age of 15.9 months, is obtained for the carcinomas and an incidence of 30 percent, with a mean tumor age of 17.1 months, for the sarcomas. The animals that had both tumors at different sites (rows 7 and 8 of table 3) are included in both incidence figures.

Strain C3Hb is known to have a low incidence of lymphoid tumors (leukemia) of the order of magnitude of a few percent. Chronic irradiation to 8.8 r daily (8 hours) elicits under the conditions of this experiment a lymphoid tumor incidence of 36 percent, with a mean tumor age of 13.5 months. As it is impossible to determine the onset of the disease, the mean tumor age given is based upon the time of autopsy. The occurrence of leukemia tabulated according to the lymphatic structure involved is recorded in table 3.

The data indicate that in the majority of cases the thymus was involved. However, the histologic types of lymphoid tumors are quite variable. Beside the variation in the number of structures involved there were also considerable differences in the degree of secondary infiltration in other organs. This is in agreement with data on incidence of lymphoid tumors observed following acute exposures to roentgen radiation.

The data on ovarian tumors are compiled in table 4. The classification of the ovarian tumors induced by irradiation described by Furth and Butterworth (*3*) will be used to facilitate comparison of the currently pre-

TABLE 2.—*Mammary tumor incidence in chronically irradiated C3Hb mice*

Age in months	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	Total	Incidence (percent)	Mean tumor age (mo.)
Number autopsied	2	3	6	5	10	12	7	9	6	13	9	5	1	2	1	91		
Adenocarcinoma			1	1		4				1	1	1	1	1		11	12	15.3
Adenoacanthoma				1	1											2	2	11.5
Carcinosarcoma						2				1						3	3	14.3
Sarcoma							1	5	4	6	4	1		1		22	24	16.7
Adenocarcinoma and sarcoma										1		1			1	3	3	19.3
Adenoacanthoma and sarcoma											1	1				2	2	18.5
No mammary tumors	2	3	5	3	9	6	6	4	2	4	3	1				48		

TABLE 3.—*Occurrence of lymphoid tumors (leukemia) of chronically irradiated C3Hb mice according to lymphoid structures involved*

Age in months	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	Total
Number autopsied	2	3	6	5	10	11	7	9	6	13	9	5	1	2	1	91
Thymus					1	1										2
Thymus and lymph nodes					1											1
Thymus, lymph nodes, and spleen	1	2	1	2	1	3	3	3	1	2	1					20
Lymph nodes and spleen			3		1		1	1		3					1	10
No lymphomas	1	1	2	3	6	8	3	5	5	8	8	5	1	2		58

TABLE 4.—*Occurrence of ovarian tumors in chronically irradiated C3Hb mice*

Age in months	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	Total
Number autopsied	2	3	6	5	10	12	7	9	6	13	9	5	1	2	1	91
Granulosa-cell tumors			1	1	3	3	4	8	4	11	5	3	1	1	1	46
Luteoma	1								1							2
Tubular downgrowth	1	2	5	2	3		1									14
Tubular adenoma						1					3					4
Luteoma and tubular adenoma						1										1
Granulosa-cell tumor and luteoma											1					1
Granulosa-cell tumor and tubular adenoma				1	1		2		1	1						6
Granulosa-cell tumor and luteoma and tubular adenoma						5				1						6
No data [1]		1		1	3	2		1				2		1		11
No tumors																

[1] Histologic section not good enough for an unequivocal diagnosis.

sented data with other data. Following disappearance of the follicles from the ovary, consistently invaginative tubular downgrowth of the surface epithelium was found. When such downgrowths had proliferated to the stage at which their mass equaled or exceeded that of the size of a normal ovary, they were tabulated as tubular adenomas. A second type of tumor was the luteoma, and a third type the granulosa-cell tumor. To facilitate analysis of the data, two further subdivisions have been used; mixed tumors with or without granulosa-cell components.

As ovarian tumors rarely kill the animals, the time of occurrence given in the table is the time of autopsy. The incidence of ovarian tumors was found to be 88 percent, which included only those cases in which an unequivocal diagnosis could be made. It is of interest to note that the majority of the tumors observed were granulosa-cell tumors and those containing granulosa-cell elements. According to Furth and Butterworth (*3*), such tumors secrete hormones. Their connection with the mammary tumors will be discussed on page 958.

Aside from the tumors described, other neoplasms were observed. Of these, the pulmonary tumors and hepatomas show an incidence greater than that of the controls. Heston's (*1*) data indicate that in his 188 breeders the lung-tumor incidence was 2.7 percent and the hepatoma incidence 4.8 percent. The lung-tumor incidence in the chronically irradiated C3Hb mice was 14 percent and the hepatoma incidence 8 percent. The over-all incidence of hepatomas, however, is not greater than the incidence of 9.4 percent found by Andervont (*8*) in C3H females without the milk agent when over 15 months of age. The data given are merely qualitative as both tumors rarely kill the animals. Both tumors are diseases of old age. The significance that these tumors were induced by irradiation is increased by the fact that the mean-survival time of the irradiated animals was considerably shorter than that of breeders.

DISCUSSION

The results of this experiment confirm previous findings on mice chronically exposed to gamma radiation under conditions identical with those of the present experiment. Although data are available on only LAF_1 hybrids, it seems that they allow general conclusions as to survival, hematopoietic effects, and carcinogenesis. The mean survival time of the C3Hb mice, after the beginning of the exposure, was found to be 405± 10 days, with a mean accumulated dose of 3,570 r; while the mean survival of LAF_1 mice was 488± 11 days, with a mean accumulated dose of 4,300 r. Control C3Hb mice had a mean survival time of 684± 11 days and control LAF_1 mice a mean-survival time of 703± 23 days. In the data of the LAF_1 mice, males and females are considered together, since no sex difference in survival was found. In comparing the survival data, it must be considered that the exposure of C3Hb mice was begun at the age of 1 month, when the animals were not mature, while irradiation of the LAF_1

mice was started at maturity (approximately 3 months). This age difference may be responsible for the earlier death of the exposed C3Hb mice.

At the chronic dose of 8.8 r daily (8 hours), the death of the mice is in part due to the carcinogenic action of the irradiation and in part due to as yet unknown causes. The carcinogenic action of whole-body irradiation seems to be to increase the incidence and decrease the tumor-development time of spontaneously occurring tumors. So far, no tumor has been observed following whole-body chronic irradiation that has not been observed spontaneously. Moreover, evidence is mounting to indicate that systemic effects of the irradiation may play a more important role in carcinogenesis than is generally assumed. Most of the evidence for a systemic action has been brought forth in the induction of leukemia in mice (*9*, *10*). The findings of the present experiment, that carcinomas and sarcomas are induced at the site of the mammary gland, also point toward a systemic effect. This is, however, not yet conclusive.

The induction of mammary tumors in chronically irradiated virgin mice is the most interesting outcome of the experiment. Heston *et al.* (*1*) found in 160 virgin mice of the same strain, nursed on C57 black females, a mammary-carcinoma incidence of 4 percent. However, when C3Hb females were intensely bred, 38 percent out of 188 females developed mammary carcinomas, with an average tumor age of 20.3 months. Tests for the milk agent by preparing cell-free filtrates from the tumors and injecting them into susceptible female mice, 1 to 5 days of age, failed to show the presence of the milk agent. The fact that the intensely bred mice developed mammary carcinomas has been explained by Heston as follows: Many factors both genetic and nongenetic may be influential. Furthermore, even if one of the factors such as the milk agent is missing, the preponderance of another factor may be sufficient to carry over the threshold of tumor formation. In intensely bred C3Hb females this other factor is hormonal stimulation, hence, the increase in mammary tumor incidence over that of the virgins. In the breeders, the tumors were mainly adenocarcinoma and adenoacanthoma with but 1 sarcoma of the mammary-gland region and 1 carcinosarcoma; in the irradiated virgin animals the sarcomas predominated, the sarcoma incidence being 30 percent, with a mean tumor age of 17.1 months, and the adenocarcinoma and adenoacanthoma incidence being 20 percent, with a mean tumor age of 15.9 months. The finding of sarcomas, adenocarcinomas, adenoacanthomas, and carcinosarcomas of the mammary gland may lead one to consider that these histologically different tumor types have a very similar etiologic factor. On the other hand, by the painting of agent-free dba mice with methylcholanthrene (*7*), Andervont induced the same type of tumors as were found in irradiated mice. No ovarian tumors were observed grossly in the painted dba mice. Feeding of methylcholanthrene or dibenzanthracene produced atrophy of the ovaries (*10*, *11*). However, since the amounts of methylcholanthrene ingested is far in excess of the amounts used in

painting, it is possible that ovaries of painted animals are more or less normal. It can be assumed, therefore, that the tumors in the dba animals were not caused by excessive hormonal stimulation as in the C3Hb breeders, but rather by a direct action of the methylcholanthrene absorbed through the skin upon the mammary gland. Of course, it can also be argued that methylcholanthrene in the small amounts present in the animal after painting may act like a hormone.

What is the mechanism by which chronic irradiation induces tumors of the mammary gland? Previous experiments (*4*) have shown that the sterilization dose for strain C3H female mice (carrying the milk agent) is approximately 500 r when exposed chronically to 4.4 r daily (24 hours). There is no reason to believe that the sterilization dose for C3Hb mice is different from that of C3H mice. From other experiments (*5*) we are justified in assuming that the sterilization dose for chronically irradiated mice is independent of the dose rate and that only the total accumulated dose is important. Hence, C3Hb mice exposed to 8.8 r daily (8 hours) are probably sterile after 2–3 months of exposure. Although sterilization by irradiation does not mean cessation of the production of estrogenic hormones, the amount produced will certainly be less than that of normal virgins. Later in life the irradiated animals develop ovarian tumors, the first tumors being observed at 8 months of age. While the early tumors were either luteomas, or tubular adenomas, the later tumors beginning at 10 months of age were predominantly granulosa-cell tumors or mixed tumors containing granulosa-cell elements.

According to Furth (*2, 3*), these ovarian tumors produce estrogen-like hormones. The possibility then exists that the mammary carcinomas were induced by hormonal stimulation. However, between sterilization and formation of ovarian tumors there was a time interval of a number of months in which estrogen production was probably low, yet mammary carcinomas appeared earlier in the irradiated mice than in mice intensely bred beginning at maturity. One can assume, of course, that the granulosa-cell-type tumors produce such large amounts of estrogen (for which histologically there is no proof) that the tumors appear in spite of a period of low estrogenic activity. On the other hand, it is also possible that other unknown factors may play a role in the radiation genesis of the mammary carcinoma and adenoacanthomas. It is possible that a combination of factors is responsible like the direct effect of the irradiation on the mammary gland in addition to the effect of the granulosa-cell tumor or unknown systemic effects.

Like the mammary carcinomas, the mammary sarcomas are also associated with granulosa-cell tumors. They were rare in the control C3Hb animals and also rarely occur in other strains of mice. The genesis of these tumors is obscure. As the earliest sarcomas were observed at the age of 13 months, a time at which the granulosa-cell-type tumors are well

established, the possibility exists that an unknown steroid produced by the ovarian tumor may elicit the sarcoma.

Some preliminary experiments are under way to attempt to elucidate the role of granulosa-cell tumors of the ovaries in the induction of mammary tumors. Granulosa-cell tumors arising in irradiated C3Hb mice have been carried as transplants in nonirradiated C3Hb normal and castrated male and female mice. No sarcomas at sites of the mammary glands have so far appeared.[2] The rapidity of growth and the superficial ulceration of the subcutaneously growing granulosa-cell tumor, however, result in early death of such mice (6 to 10 months after transplantation), which might mask our observation of the significance of this factor even though existent. The induction of the mammary sarcomas may also be a combined effect of an unknown steroid and a direct effect of the radiation on the mammary gland. It seems, however, unlikely that a direct effect of the irradiation on the mammary gland alone would be responsible in the induction of the mammary tumors. Newborn C3Hb mice [3] exposed to an acute dose of roentgen radiation of 400 r developed but one mammary carcinosarcoma, although some have survived 21 months and a high percentage of ovarian tumors of the granulosa-cell type were observed. It can be argued that the exposure at birth destroyed the very small amount of mammary tissue present at birth. However, newborn C3H mice carrying the milk agent exposed to the same dose did develop mammary carcinomas. Therefore, irradiation at birth does not seem to destroy the mammary gland. Moreover, LAF_1 mice exposed during their entire life span to 0.11 r daily (8 hours) did also develop mammary sarcomas (table 1). The total accumulated dose at the time the tumors were observed was 80 r to 150 r. It seems improbable that a chronic daily dose of 0.11 r could have a direct effect on the mammary gland.

A few tests for the presence of the milk agent were also made, using the procedure outlined previously. They failed to show the presence of the agent. Therefore, irradiation does not seem to activate a weak agent or destroy an inhibitor. Additional experiments are under way that may clarify the genesis of these tumors.

SUMMARY AND CONCLUSIONS

Experiments were performed in which virgin C3Hb mice were chronically irradiated over the total body with 8.8 r daily (8 hours), beginning at 1 month of age and continuing throughout their entire life span.

Mean survival time of the irradiated animals was shortened by 36 percent in comparison to nonirradiated controls.

The injurious action of the irradiation was evidenced by a considerable reduction in weight after 1 year of age.

[2] Eschenbrenner: Unpublished data.
[3] Unpublished data.

The effect of the chronic irradiation on the picture of the circulating blood was surprisingly small, although the mean accumulated dose was 3,510 r and the highest accumulated dose was 5,540 r; only the lymphocytes show a pronounced drop in numbers for the greater part of the duration of the experiment.

Carcinogenesis was the predominant picture of radiation injury. Forty-seven percent of the animals developed tumors (carcinomas and sarcomas) of the mammary gland; ovarian tumors were present in 88 percent of the animals, and lymphoid tumors were observed in 36 percent. Pulmonary tumor and hepatoma incidences were likewise increased in the experimental animals in comparison to nonirradiated controls.

The following conclusions can be drawn:

1) Chronic irradiation acts similarly to hormonal stimulation in that it increases the incidence of mammary tumors in agent-free mice.

2) Although the irradiated animals were virgins and became sterile, hormonal stimulation cannot be excluded as a causative factor in the genesis of the mammary tumor because all mammary tumors were associated with a granulosa-cell tumor of the ovary.

3) The activation of a weak milk agent or destruction of an inhibitor by the irradiation seems unlikely, as tests failed to show the presence of an agent in the mammary tumor.

4) A direct effect of the chronic irradiation on the mammary gland alone seems also unlikely because chronic exposure of LAF_1 females to 0.11 r daily (8 hours) will elicit mammary sarcomas, although total accumulated doses did not amount to more than 80 to 150 r.

5) The mammary-tumor-inducing action of chronic whole-body irradiation may be caused by a combination of effects such as hormonal stimulation and direct effects upon the mammary gland or by other unknown effects of systemic origin.

REFERENCES

(1) Heston, W. E., Deringer, M. K., Dunn, T. B., and Levillain, W. D.: Factors in the development of spontaneous mammary gland tumors in agent-free strain C3Hb mice. J. Nat. Cancer Inst. 10: 1139–1151, 1950.

(2) Furth, J., and Furth, O. B.: Neoplastic diseases produced in mice by general irradiation with x-rays. Am J. Cancer 28: 54–65, 1936.

(3) Furth, J., and Butterworth, J. S.: Ovarian tumors and associated lesions. Am. J. Cancer 28: 66–95, 1936.

(4) Lorenz, E., and Heston, W. E.: Biologic effects of long continued total body gamma irradiation on mice, guinea pigs, and rabbits. Part I, NNES, Div. IV, vol. 22B. In preparation.

(5) Lorenz, E., Heston, W. E., Jacobson, L. O., Eschenbrenner, A. M., Shimkin, M., Deringer, M. K., and Doniger, J.: Biologic effects of long continued total body gamma irradiation on mice, guinea pigs and rabbits. Part III, NNES, Div. IV, vol. 22B. In preparation.

(6) Lorenz, E.: Biologic effects of long continued total body gamma irradiation on mice, guinea pigs and rabbits. Part II, NNES, Div. IV, vol. 22B. In preparation.

(7) ANDERVONT, H. B., and DUNN, T. B.: Response of mammary-tumor-agent free strain dba female mice to percutaneous application of methylcholanthrene. J. Nat. Cancer Inst. 10: 895–925, 1950.

(8) ANDERVONT, H. B.: Studies on the occurrence of spontaneous hepatomas in mice of strains C3H and CBA. J. Nat. Cancer Inst. 11: 581–592, 1950.

(9) KAPLAN, H. S.: Local irradiation and the induction of lymphoid tumors in mice. Presented at the fortieth annual meeting of American Association Cancer Presented at the fortieth annual meeting of American Association for Cancer Research Abstract. Cancer Research 9: 621, 1949.

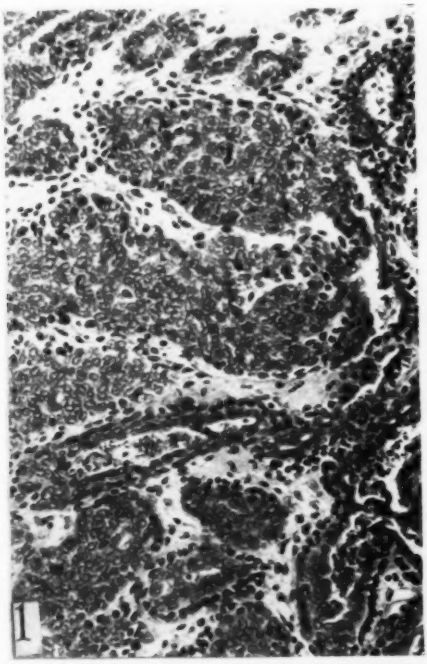
(10) LORENZ, E.: Some biologic effects of long continued radiation. Am. J. Roentgen. & Radium Ther. 63: 176–185, 1950.

(11) LORENZ, E., and STEWART, H. L.: Intestinal carcinoma and other lesions in mice following oral administration of 1,2,5,6-dibenzanthracene and 20-methylcholanthrene. J. Nat. Cancer Inst. 1: 17–40, 1940.

(12) ———: Tumors of the alimentary tract induced in mice by feeding olive oil emulsions containing carcinogenic hydrocarbons. J. Nat. Cancer Inst. 1: 227–238, 1947.

PLATE 100

FIGURE 1.—Mammary adenocarcinoma. Hematoxylin and eosin stain. × 200
FIGURE 2.—Adenoacanthoma. Hematoxylin and eosin stain. × 250
FIGURE 3.—Mammary carcinosarcoma. Hematoxylin and eosin stain. × 150
FIGURE 4.—Mammary carcinosarcoma. Wilder reticulum counterstained with hematoxylin. × 250



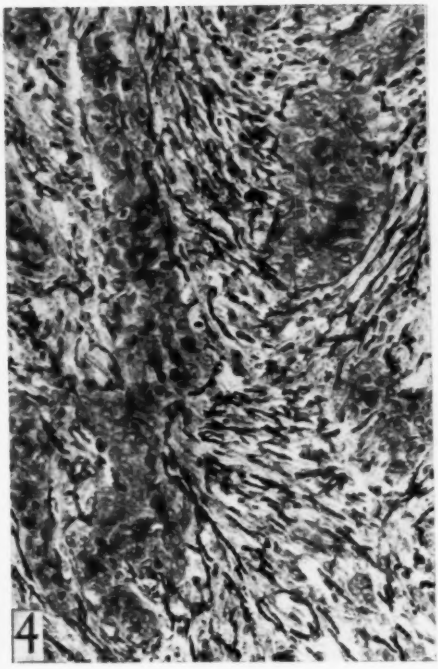


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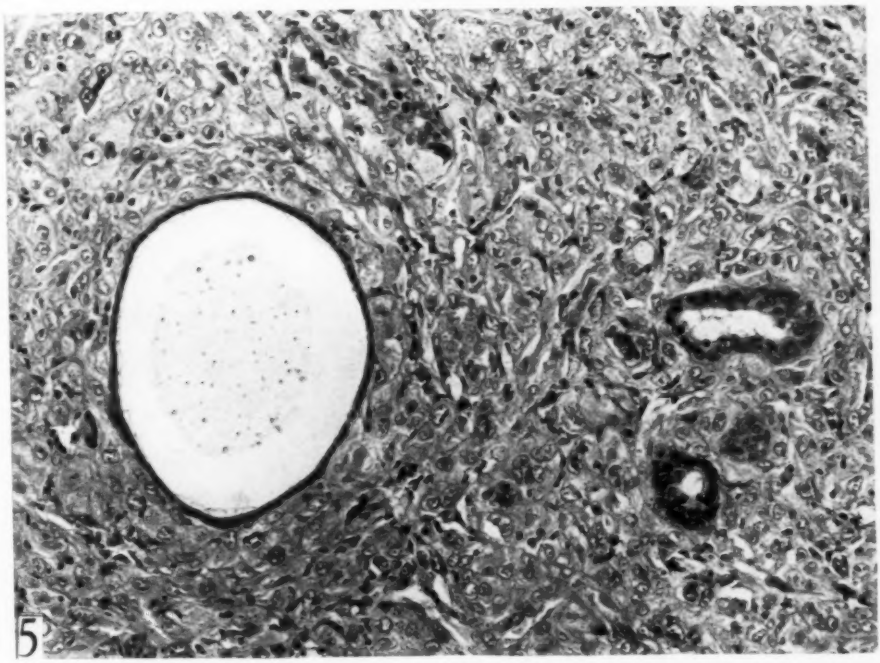
PLATE 101

FIGURE 5.—Mammary sarcoma incorporating ducts of mammary gland. Hematoxylin and eosin stain. × 250

FIGURE 6.—Mammary sarcoma incorporating ducts of mammary gland and associated adipose tissue. Hematoxylin and eosin stain. × 250



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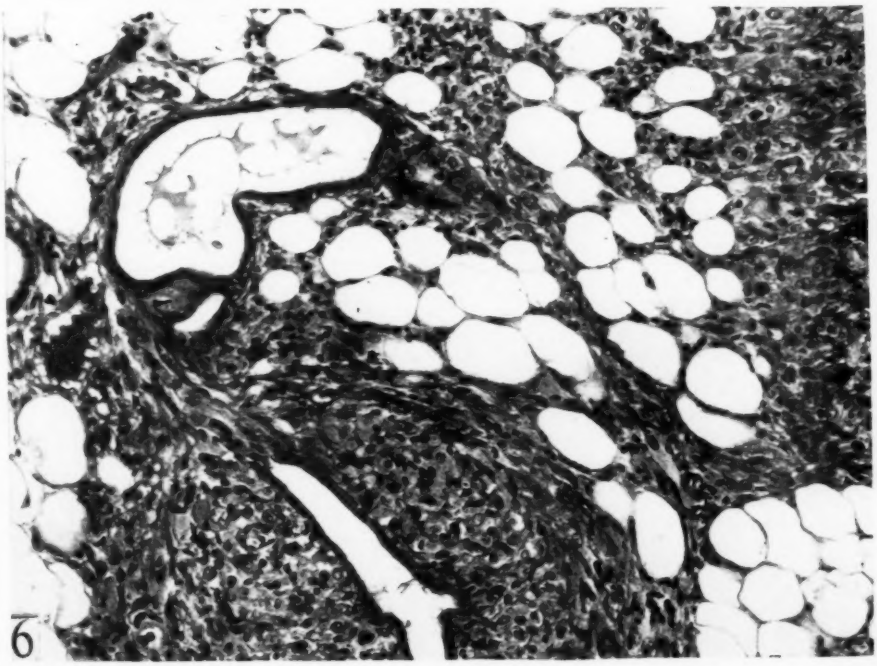

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IRRADIATION OF THE NORMAL HUMAN HYPOPHYSIS IN MALIGNANCY: REPORT OF THREE CASES RECEIVING 8,100–10,000 r TISSUE DOSE TO THE PITUITARY GLAND[1,2]

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Certain malignant neoplasms of man, particularly carcinoma of the prostate and of the mammary gland, are under some degree of control by the endocrine secretions of the host. Partial regression of such neoplasms is achieved by changing the hormonal status of the host, such as by castration or by administration of steroid hormones (*1*, *2*). The possible role of endocrine influences in malignant melanoma is also suggested by the intriguing observations that melanomas in man are rarely malignant before puberty (*3*) and are not observed in castrated individuals (*4*).

The mode of action of castration or steroid hormones in inhibiting the growth of mammary and prostatic carcinoma has been a topic for considerable speculation. One of these speculations is that the action is exerted through alterations in pituitary function (*2*). This hypothesis has not been studied directly in man, and the lack of proper experimental animals and tumors in which similar effects can be reproduced has prevented definitive laboratory investigations. It was therefore decided to attempt reduction of pituitary function in patients by means of irradiation of the pituitary gland.

Lawrence, Nelson, and Wilson (*5*) have summarized the work on pituitary irradiation in animals; they quote 15 articles, starting with the investigations of Strauss and of Brunner in 1920. Direct comparison of the results is not possible because of variance in dosage of irradiation, species and age of the animals. In general, however, it may be concluded that the pituitary gland is relatively radioresistant. Loss of weight, de-

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[4] Fellow, Canadian Government Educational Program.

[5] Fellow, American Cancer Society.

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crease in the size of the ovaries, testes and other endocrine organs, and histologic evidence of degenerative changes in the pituitary gland are reported only in young or immature animals, whether they be rats, rabbits, cats, or dogs. In contrast, adult animals have shown little or no discernible effects following irradiation of the pituitary gland. Podljaschuck (*6*), for example, reported that doses equivalent to approximately 500 r pituitary-tissue dose produced no changes in adult dogs, whereas doses above the equivalent of 700 r in puppies were followed by marked histologic changes in the pituitary gland, atrophic changes in the ovaries, and retardation of growth and weight gain. Lacassagne (*7*) irradiated mature rabbits, delivering approximately 5,000 r pituitary-tissue dose by single exposures through 2 relatively large fields. There were loss of weight and decrease in the size of the testes and ovaries, but the pituitary glands showed no gross or microscopic changes. Fractional doses of as much as 8,000 r pituitary-tissue dose had no effect, and 3 to 5 months later the examination of the pituitary gland revealed no abnormalities. Direct implantation of radon seeds into the pituitary fossa destroyed the pituitary gland, but if one-third of the anterior lobe remained intact, genital function was not impaired.

Radiation therapy to the pituitary gland has had wide clinical use in patients with pituitary tumors. Doses up to 4,500 r to the tumor have been advocated. Pituitary irradiation has also been employed in essential hypertension, amenorrhea, and certain other gynecologic disorders, diabetes mellitus, and thyrotoxicosis. The results in these conditions are difficult to interpret, and almost all favorable reports suffer from either lack of objective criteria of improvement or adequate control groups of patients. This topic is ably summarized by Menville (*8*). Best *et al.* (*9*) irradiated the pituitary gland in 25 patients with hypertensive vascular disease, delivering a tissue dose of 1,100 r in 10 days. No effects were observed, and examination of 1 pituitary 11 months after treatment showed no deviation from normal. For the purpose of this investigation, it is sufficient to note that the doses of radiation used clinically do not exceed 2,000 r pituitary-tissue dose, and that no untoward effects are noted with such procedures.

On the basis of the above information, doses considerably higher than applied previously in the therapy of intracranial lesions were considered necessary in order to achieve reduction or interruption of pituitary function. This required the development of a method of delivering a very high radiation dose to the pituitary gland with minimal irradiation of the surrounding structures.

EXPERIMENTAL PROCEDURE

An apparatus (fig. 1) was constructed permitting irradiation of the pituitary gland through multiple small fields of entrance. The patient's head was fitted into a plaster-cast skull cap surmounted by a plate into which a bolt was incorporated. The bolt was adjusted to be in the axis of the pituitary fossa, and attached to a frame projecting from a standard X-ray

machine base plate. The X-ray beam was adjusted to be perpendicular to the axis of the bolt, and passed through the pituitary fossa at any position of the head rotating around the bolt axis. The beam size was defined by two lead diaphragms attached to the projecting arms, one at the base plate of the X-ray tube and one close to the patient's head.[6]

The patient was seated upright in a rigid but comfortable position. Repeated radiographic examinations showed that the position of the beam relative to the sella turcica did not vary more than one millimeter for any field of entrance after the initial adjustments were made.

Figure 2 shows the convergence of the beam on a film placed in the axis of the X-ray beam in a head-shaped paraffin phantom at the level of the pituitary fossa. The 10 surrounding views are spot films made on the therapy machine and correspond to the radiation fields employed in Case 1.

The physical factors of irradiation were: 200 KV; 1.2 mm. Cu HVL; 2.5-cm. diameter field size; tube target-pituitary distance varied from 63 to 70 cm.; defined field size at the pituitary 2.7 cm. in diameter. Daily doses of 400 r measured in air were given to one, two or three fields; usually two fields were used. The dose calculation on the skin portals refers to total skin dose including the exit dose from opposing fields.

The surface, depth, and exit doses were determined for each portal, using a pressed-wood phantom and a 25 r Victoreen ionization chamber. A human skull filled with rice and the orbits and paranasal cavities with wet cotton also was used as a phantom. A 25 r Victoreen ionization chamber was placed in the pituitary fossa. The calculated tissue dose and the experimentally determined tissue dose were in good agreement, being within 10 percent for the nasion field and not more than 3 percent for all other fields.

Three patients with far-advanced neoplastic disease were selected for the investigation. All 3 patients had complete clinical studies performed before, during, and after the completion of irradiation to the pituitary gland. These included determinations of the visual fields and audiographs. Special studies designed to reveal evidence of physiologic effects included determinations of the basal metabolic rate on the Sanborn respirometer, serum iodine by the method of Sandell and Kolthoff, blood levels of albumin and globulin, sodium and potassium by flame photometry, chloride by the method of Schales and Schales, calcium by the method of Kramer and Tisdall, phosphorus by the method of Hawk and Summerson, alkaline phosphatase by Bodansky's technique, and free and total cholesterol by the method of Schoenheimer and Sperry. Blood bilirubin and nonprotein nitrogen levels were also determined. Glucose tolerance was studied following the intravenous administration of 25 grams of glucose. Twenty-four-hour excretion of 17-ketosteroids in the urine, and 24-hour excretion of pituitary gonadotropins by a modified Scott's bioassay technique were also measured, the results of the latter being considered normal if positive at a dilution of 1 to 5 and negative at 1 to 80.

[6] Detailed report of the physical principles involved in this type of directed beam therapy will appear elsewhere (Low-Beer, B. V. A., Feldsted, E. T., and Brown, R. F., in preparation).

Case 1 (M. E. K.—LEO 83)

This 60-year-old white woman had disseminated adenocarcinoma of the right breast. At the age of 55 she had noted a painless nodule in the right breast. Radical mastectomy was performed 2 months later, and she remained clinically free of disease until 2 months before admission. Recurrence was first noted in the right supraclavicular region, followed by the appearance of hard, nontender subcutaneous nodules along and near the surgical scar.

The patient had had 10 spontaneous abortions without known cause, and 2 normal deliveries. She underwent the menopause at the age of 48.

Physical examination showed a well developed, somewhat obese, white woman, The right breast was absent, and there were over a dozen hard subcutaneous nodules along the operational scar as well as the anterior chest wall. The supraclavicular space was filled with a hard, fixed mass measuring approximately 6 cm. in diameter. Roentgen examination failed to reveal osseous or pulmonary metastases. Biopsy of one of the subcutaneous nodules revealed metastatic adenocarcinoma compatible with the histologic appearance of the primary carcinoma of the breast.

Irradiation directed to the pituitary fossa was started on April 22, 1949. The patient was treated through 10 fields, centering and crossing at the pituitary fossa. She received 47 treatments in 62 days. The skin dose to each field varied from 3,000 r to 3,800 r, due to the change in TSD resulting from the oval shape of the skull. The total dose calculated at the pituitary was 8,150 r.

The patient tolerated the procedure without complications. Epilation and tanning of the skin developed over the radiation portals. Her clinical status was determined before and at three periods during therapy, as well as 2 months after its completion. All special clinical and laboratory determinations remained within normal limits, and no evidence was elicited that pituitary irradiation produced hypopituitarism or other effects.

Measurements and clinical observations of the metastatic lesions did not reveal any effects on the tumor. She moved to St. Louis, and her progress has been followed by Dr. Edward H. Reinhard. In October 1949, 4 months after pituitary irradiation, the lesions had increased in size, and she was given local roentgen treatments to the individual nodules, with regressions. In May 1950, she developed severe pain in the right lower chest. No metastases were demonstrated roentgenologically but marked improvement followed treatment with testosterone propionate. A year following completion of pituitary irradiation the patient feels well and has continued to work.

Case 2 (G. M. H.–LEO 114)

This 38-year-old white woman had disseminated melanoma arising from a plantar pigmented mole. She had had a pigmented mole on the plantar surface of the fourth left toe "since birth." Six years ago, 4 months after the onset of her second pregnancy, there was a change in the appearance of the lesion, and the obstetrician amputated the toe. The diagnosis on microscopic examination was malignant melanoma. Three weeks following amputation of the toe, a lymph-node dissection was made of the left inguinal region, but no metastases were demonstrated. Three months later a black nodule appeared on the inner surface of the left thigh; biopsy showed this to be a melanoma. She was allowed to continue pregnancy, and a normal child was delivered at term.

New melanotic nodules appeared on the left thigh and lower left leg, and edema of the left extremity became progressively more marked.

Physical examination on admission revealed a well nourished, well developed woman. The left leg was swollen with edema, and there were numerous melanotic nodules, some measuring up to 2 cm. in diameter, over the whole extremity. The left inguinal region was filled with firm, nontender masses extending above the inguinal ligament.

Irradiation directed to the pituitary fossa was started on September 17, 1949. The patient was treated through 10 fields, centering and crossing at the pituitary fossa. She received 50 treatments in 63 days. The skin dose to each field varied from 3,300 r to 4,100 r, due to the change in TSD resulting from the oval shape of the skull. The total dose calculated at the pituitary was 8,516 r.

Clinical and laboratory studies, as in Case 1, were performed on the patient before, during, and after irradiation. There was slight nausea during treatment, and the only effects observed in the laboratory determinations were a slight elevation of free and total cholesterols and a slight increase in the urinary output of 17-ketosteroids. All determinations, however, were well within normal limits.

The melanotic lesions on the left leg increased steadily in size, and many became ulcerated. There were progressive weakness and loss of weight. Ascites appeared approximately 2 months following the completion of irradiation, and melanin began to appear in the urine. The patient died exactly 3 months following completion of irradiation to the pituitary gland. Terminally there were sharp rises in blood uric acid and nonprotein nitrogen.

Autopsy, 8½ hours after death, showed massive involvement of the left extremity with melanoma, which was predominantly amelanotic on microscopic examination. There were distant metastases to skin, peritoneal and pleural surfaces, lungs, liver, spleen, gastrointestinal tract, lymph nodes, internal genitalia, and breast. The pituitary gland measured 1½ by 1 by ½ cm. and weighed 1.1 grams. The dura over the gland was thin, and it had a normal gross appearance. Examinations of all the structures at the base of the brain, including the bone itself, failed to reveal any abnormalities. The right adrenal gland weighed 11 grams, the left, 14 grams, and both were of normal size and shape. The thyroid gland weighed 14 grams and showed only pallor. Examination of the internal genitalia was obscured by tumor growth replacing both ovaries; however, the uterus was within normal limits.

Microscopic examination of the pituitary gland showed a delicate, well formed anterior and posterior lobe without cellular or vascular abnormalities. The ratio of cells within the anterior lobe appeared to be within normal limits. Examination of all the endocrine glands showed no abnormalities except where tumor was invading. Both ovaries were replaced by tumor. The endometrium of the uterus was scanty and showed a few small glands. Examination of the base of the brain, internal carotids, pineal body, cranial nerve ganglia, optic chiasm, and floor of the third ventricle was negative. The tumor appeared to be well preserved, and there was no fibrosis or shrinkage of tumor cells.

Case 3 (J. C.–LEO 124)

This 59-year-old white woman had disseminated bilateral adenocarcinoma of the breast. Four years previously she noted a painless lump in her left breast. A radical mastectomy was performed. Approximately 18 months later small, hard, subcutaneous nodules appeared near the incision line, and a separate hard mass was found in the right breast. Roentgen therapy was administered over the anterior chest and right breast, with some regression of the size and number of the subcutaneous nodules. Her weight remained stationary at about 140 pounds until 6 months before entry, since which time she lost approximately 10 pounds in weight.

For about 20 years she had had high blood pressure, and for the past year had irregularly taken digitalis. She had two pregnancies, one terminating in a spontaneous abortion, and had passed the menopause at the age of 50.

Physical examination showed a well developed, well nourished woman, with a hoarse voice and some dyspnea on recumbent position. The right breast was stony hard, not tender, and the mass was fixed to the ribs. The left breast had been amputated, and along the surgical scar and surrounding subcutaneous tissue were approximately 10 hard, flat nodules, each measuring 3 to 6 cm. in diameter. The right axillary lymph nodes were enlarged, but soft; no lymph nodes were felt in the left axilla. Laryngoscopic examination showed the left vocal cord to be paralyzed.

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The heart was of normal size, and regular in rate and rhythm. The blood pressure was 180/110. The lungs were clear. There was slight pretibial edema bilaterally, but the patient had varicosities of both lower extremities.

Biopsy of a subcutaneous nodule revealed anaplastic adenocarcinoma. Roentgen films of the chest showed increased bronchovascular markings and an elevated diaphragm on the right side. Electrocardiograms were within normal limits. The basal metabolic rate was +24.

Irradiation directed to the pituitary fossa was started on November 17, 1949. The patient was treated through 10 fields, centering and crossing at the pituitary fossa. She received 47 treatments in 62 days. The skin dose to each field varied from 4,200 r to 4,800 r, due to the change in TSD resulting from the oval shape of the skull. The total dose calculated at the pituitary gland was 10,049 r.

She was readmitted for study 1 month following the completion of radiation therapy. Her weight had declined by about 20 pounds, but otherwise she felt as well as before therapy. There had been no alteration in the size or extent of the neoplasm, and all laboratory and clinical findings indicated no significant changes. The basal metabolic rate was +17. The blood pressure was 130/90.

Three months following pitutary irradiation the patient developed clinical evidence of cardiac decompensation and was placed on digitoxin, a low-salt diet, and other supportive measures. The metastatic nodules had progressed in size, and the left arm became swollen with edema. She was started on methyl testosterone, 60 mg. per day for 28 days, with definite reduction in size of the metastatic nodules. However, she developed marked generalized edema, and the blood sodium fell to 120 milliequivalents per liter. These effects were promptly restored to normal by intravenous therapy with electrolytes.

The patient died 4½ months following the irradiation of the pituitary gland, with progressive weakness, cachexia, and cardiac decompensation. Terminally the blood proteins dropped to 4 gm. per 100 cc., and the red-blood-cell count to 2,000,000 per cubic millimeter.

Autopsy, 3 hours after death, showed extensive involvement of lymph nodes, mediastinum, and lungs by carcinoma. The right breast was also infiltrated with carcinoma, as well as the skin of the old left mastectomy scar. Both innominate veins were stenosed, with striking edema of the upper extremities. The pituitary gland was symmetrical and of normal size, weighing 0.8 gram and measuring 1.3 by 1 by 0.9 cm. Examination of the base of the brain, basal blood vessels, dura, and bone revealed no abnormalities. The right adrenal gland weighed 10 grams, the left 12 grams; both cortices were thick, with considerable lipoid. The thyroid gland was somewhat diminished in size, weighing 10 grams. The uterus, tubes, and ovaries showed involutionary changes, and two small myomas were noted in the uterine fundus.

Microscopic examination showed the anterior lobe of the pituitary gland to be cellular and to have a normal ratio of cells. A slight degree of interstitial fibrosis was noted. Staining with azure-eosin, Mallory's aniline-blue collagen stain and Ludlow's silver-carbonate stain failed to reveal any unusual abnormalities not compatible with the patient's age. The cerebral blood vessels showed arteriosclerosis with an old vascular lesion involving the right putamen. Scattered necrotic foci were noted in the thalamus and pons in which the tissue was spongy with little glial or vascular reaction. There was a diffuse proliferation of microglia, and macroglia, particularly in the form of the Alzheimer glial type. Histologically the adrenal, thyroid, and parathyroid glands were entirely negative. The breast, ovaries, tubes, and uterus showed only involutionary changes quite consistent with the patient's age. The endometrium was scanty, congested, and contained occasional cysts. The uterine myomas were well differentiated and had a uniform appearance. The metastatic tumor was a well preserved adenocarcinoma.

DISCUSSION

Three adult women with advanced neoplastic disease received X-ray irradiation to the pituitary gland for 2 months, for total tissue doses to the pituitary of 8,100 to 10,000 r. All three patients withstood the procedure without untoward reactions.

Clinically, no evidence was elicited that pituitary function was reduced by the irradiation. Table 1 presents the major determinations on the blood and urine of the patients before irradiation, at the completion of irradiation, and 2 months after the completion of irradiation. Considering that the three patients had progressive neoplastic disease, and that the third patient also had severe cardiac damage which eventuated in decompensation, remarkably little variation was observed in these values. There was, perhaps, slight elevation in the free and total cholesterol levels of the blood, the urinary pituitary gonadotropin output appeared to decrease in two of the three patients, and the basal metabolic rate was increased from +6 to +12 in two patients but decreased from +24 to +17 in the third at the completion of pituitary irradiation. The urinary excretion of 17-ketosteroids, however, was increased slightly, and no alteration was detected in the serum iodine. The glucose-tolerance curve following intravenous administration of 25 grams of glucose remained normal in all three patients. The reduction in the blood pressure in Case 3 is attributable to the onset of cardiac decompensation rather than to any effect of pituitary irradiation. No definite significance can be attached to any of these observations, other than that no clear evidence of altered metabolism or other effects was demonstrated. Both patients with mammary carcinoma responded clinically to treatment with androgens following the completion of pituitary irradiation.

Necropsy examination of 2 patients revealed no morphologic abnormalities in the pituitary gland or other endocrine organs, or changes in the neoplasm. Figures 3–5 and 6–8 present the histologic appearance of the pituitary gland in Cases 2 and 3, respectively. It is to be noted that in Case 3 there is some focal fibrosis suggestive of a vascular origin. The remaining portions of the stroma and the glandular elements were delicate and well preserved. In order to interpret the interstitial fibrosis in this pituitary, pituitary glands from the last 10 consecutive necropsies on patients over 60 years of age were examined for comparison. In 3, the degree of focal fibrosis and cellular changes exceeded that seen in the patient who received irradiation to the pituitary. Special stains for relative numbers of eosinophilic, basophilic, and chromophobic cells in the 2 irradiated pituitary glands showed no abnormalities.

It is evident from this investigation that the normal pituitary gland in adult human beings is resistant to high doses of X-ray irradiation. Further studies on the effect of inhibition of pituitary function in neoplastic disease appear to be dependent upon the development of surgical techniques for the removal of the pituitary gland. The negative effects with

TABLE 1.—*Clinical laboratory determinations in three patients receiving 8,100 to 10,000 r tissue dose of irradiation to the pituitary gland*

Determination	Case No.	Before therapy	At end of therapy	2 months after therapy
Urinary 17-ketosteroids (mg./24 hours)	1	5.0	9.0	10
	2	7.5	9.5	2.1
	3	6.2	15.4	8.1
Urinary pituitary gonadotropins (mouse units/24 hours).	1	+5	+5	+5
		+80	−80	−80
	2	+5	+5	+5
		−80	−80	−80
	3	+5	+5	−5
		−80	−80	
Blood glucose, fasting (mg./100 cc.)	1	79.8	73.5	89.6
	2	65.0	69.2	66.8
	3	82.5	65.2	63.8
Glucose tolerance 120 min. after 25 gm. i. v.	1	96.6	68.3	96.6
	2	98.0	90.0	81.6
	3	80.8	70.2	
Cholesterol total (mg./100 cc.)	1	176	220	224
	2	68.2	98.3	88.3
Cholesterol free (mg./100 cc.)	1	48.7	60	84.5
	2	23.8	35.4	37.3
Basal metabolic rate	1	+6	+12	
	2	+6	+12	+8
	3	+24	+17	
Serum Iodine (micrograms/100 cc.)	1	6.6	6.7	6.9
	2	6.5	6.0	
	3	6.3	6.9	6.2
Blood pressure (mm. Hg.)	1	155/85	130/70	130/80
	2	110/65	100/60	105/60
	3	180/110	130/90	130/90
Total protein (gm./100 cc.)	1	7.9	7.4	8.3
	2	6.0	5.8	5.9
	3	6.5	6.5	6.3
Albumin (gm./100 cc.)	1	3.8	1.9	3.8
	2	3.3	2.9	3.5
	3	3.7	3.7	3.2
Globulin (gm./100 cc.)	1	4.1	5.4	4.5
	2	2.7	2.9	2.4
	3	2.8	2.8	3.2
Chlorides (meq./L)	1	110	113	109
	2	106	106	97
	3	109	106	94
Sodium (meq./L)	1	138	139	131
	2	134	122	124
	3	135	131	130
Potassium (meq./L)	1	4.3	4.8	4.5
	2	4.7	4.7	5.0
	3	5.4	4.4	4.9
Calcium (mg./100 cc.)	1	9.6	10.2	
	2		8.8	9.1
	3			
Phosphorus (mg./100 cc.)	1	3.4	3.6	3.5
	2	3.4	3.6	3.9
	3	3.5	3.7	3.5
Alkaline phosphatase (Bodansky units)	1	2.0	2.2	1.4
	2	1.4	1.8	2.1
	3		4.5	5.9
N. P. N. (mg./100 cc.)	1	36	29	45
	2	21	22	19
	3	26	21	18

X-ray irradiation in doses of 8,100 to 10,000 r tissue dose to the pituitary gland also have definite and obvious implications in the evaluation of results reported in amenorrhea, hypertension, and thyrotoxicosis, in which it is claimed that doses of under 2,000 r, and often as low as 50 r, beneficially affect such clinical conditions.

SUMMARY

Two 60-year-old women with disseminated mammary adenocarcinoma and one 38-year-old woman with disseminated melanoma were given 8,100 to 10,000 r tissue dose to the pituitary gland by means of a multiple-port coronal appliance. One patient is living 1 year later, and the others died 3 and 4.5 months after completion of the irradiation. No clinical or laboratory evidences of hypopituitary function were noted. The growth of the neoplasms was not affected. Both women with mammary carcinoma responded to androgens following completion of pituitary irradiation. Gross and microscopic examination of the pituitary gland and other endocrine organs showed no definite abnormalities in the two cases coming to autopsy.

It is concluded that the normal adult human pituitary gland is resistant to X-ray irradiation in doses up to 10,000 r.

REFERENCES

(*1*) SHIMKIN, M. B., and BIERMAN, H. R.: Experimental chemotherapy of neoplastic diseases. Radiology 53: 518–528, 1949.

(*2*) Proceedings of the First Conference on Steroid Hormones and Mammary Cancer: pp. 205. Therapeutic Trials Committee, Council on Pharmacy and Chemistry, A. M. A. Chicago, 1949.

(*3*) PACK, G. T.: A clinical study of pigmented nevi and melanomas. *In* The Biology of Melanomas, Spec. Publ., N. Y. Acad. Sc. 4: 52–61, 1948.

(*4*) PACK, G. T., and LIVINGSTON, E. M.: The treatment of pigmented nevi and melanomas. Vol. III, pp. 2071–2094. *In* Pack & Livingston, Treatment of Cancer and Allied Diseases, Paul B. Hoeber, New York, 1940.

(*5*) LAWRENCE, J. H., NELSON, W. O., and WILSON, H.: Roentgen irradiation of the hypophysis. Radiology 29: 446–454, 1937.

(*6*) PODLJASCHUK, L. D.: Experimentelle Untersuchungen über die Beziehungen zwischen Hypophyse und anderen innersekretorischen Drusen. Strahlentherapie 24: 439–458, 1927; 30: 65–76, 1928.

(*7*) LACASSAGNE, A.: Uber den Einfluss von Hypophysenbestrahlungen (mit Radonspickung oder Röntgenstrahlen) auf das Ovarium. Strahlentherapie 54: 477–492, 1935.

(*8*) MENVILLE, L. J.: Radiation therapy in diseases and dysfunctions of the glands of internal secretion. pp. 648–677. *In* Pohle, E. A., "Clinical Radiation Therapy," Ed. 2, Lea and Febiger, Philadelphia, 1950.

(*9*) BEST, M. M., COE, W. S., MOORE, J. W., REED, E. S., and CLAY, H. L.: Irradiation of the pituitary gland in hypertensive vascular disease. Am. J. Med. Sc. 219: 276–280, 1950.

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PLATE 102

FIGURE 1.—An apparatus for the controlled irradiation of the pituitary gland through multiple small fields of entrance. See text.

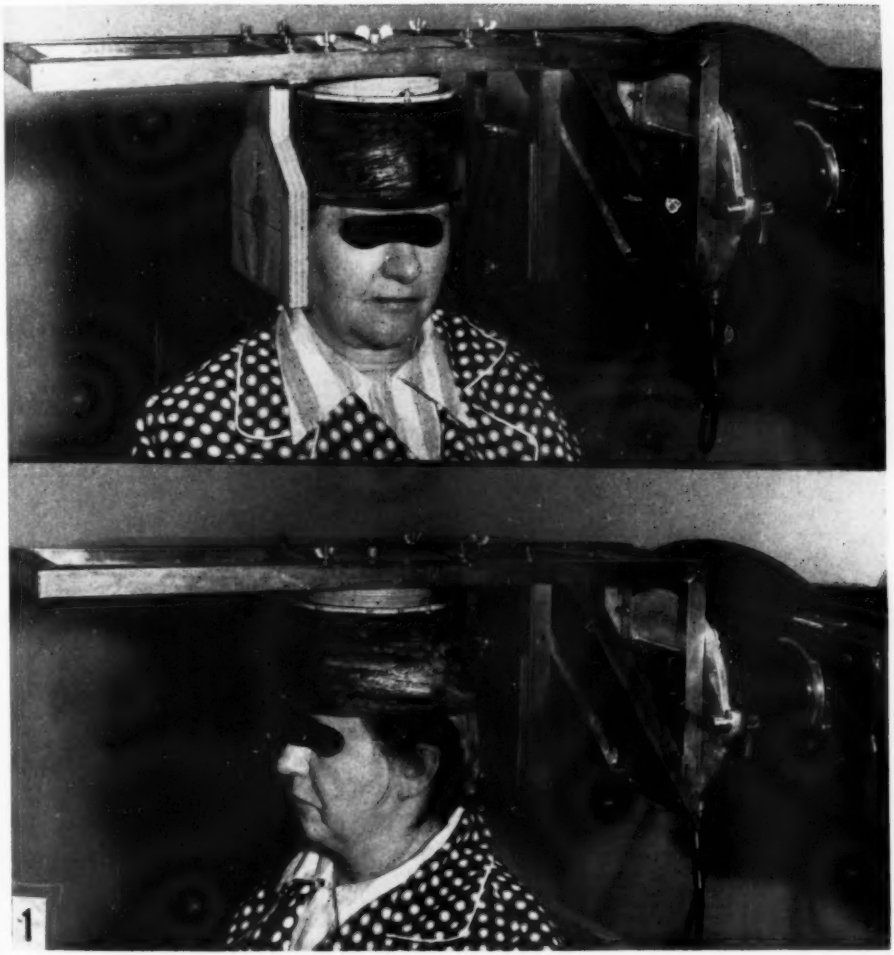

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PLATE 103

FIGURE 2.—Convergence of the beam on a film placed in the axis of the X-ray beam in a paraffin phantom at the level of the pituitary fossa. Surrounding views are spot films made through similar fields in Case 1.

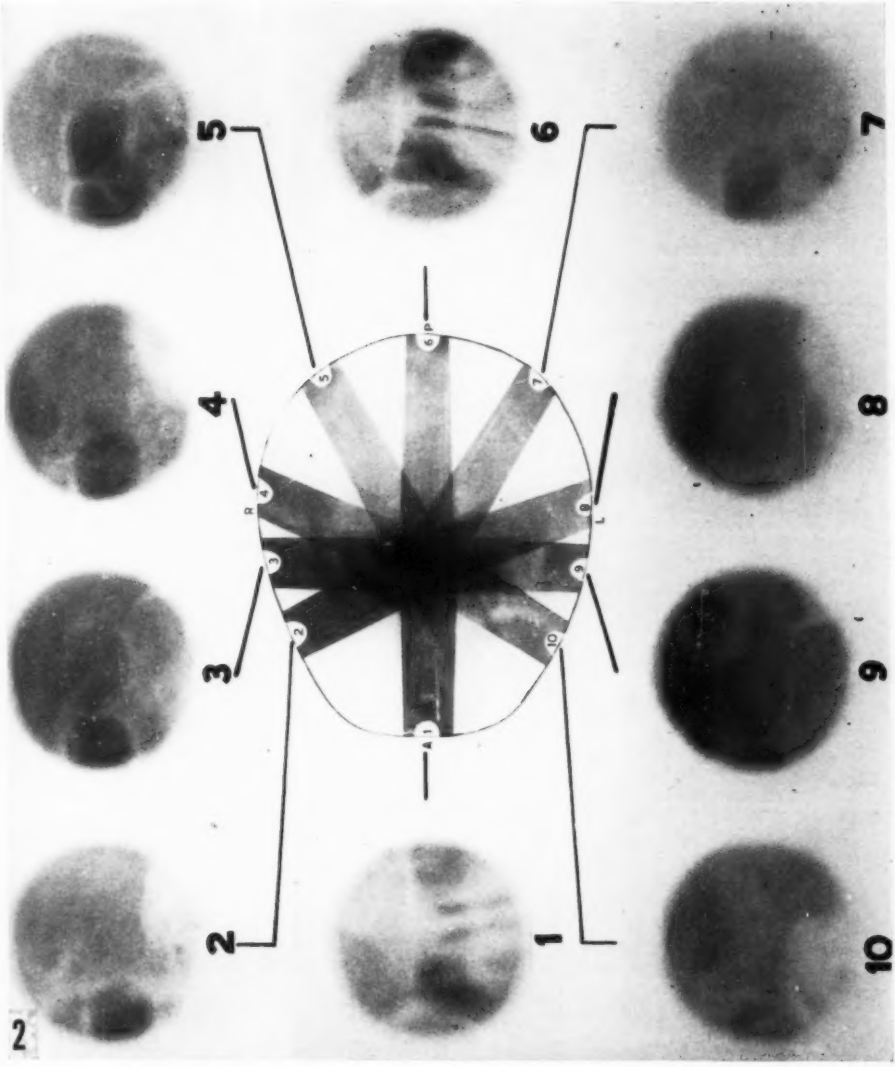
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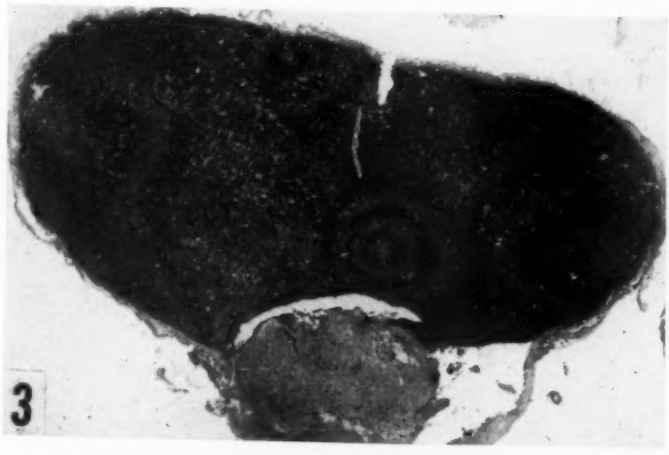
PLATE 104

FIGURE 3.—Pituitary gland in Case 2, 3 months following delivery of 8,516 r tissue dose. No gross or microscopic abnormalities were observed. Hematoxylin and eosin stain. × 10.

FIGURE 4.—Higher magnification of the pituitary gland, demonstrating the delicate blood vessels and lack of abnormalities. Hematoxylin and eosin stain. × 43.

FIGURE 5.—Cellular detail of the adenohypophysis, showing normal individual cells. Hematoxylin and eosin stain. × 100.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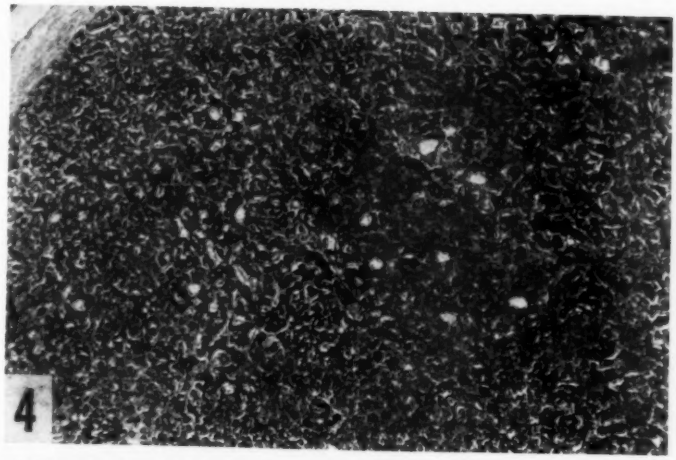
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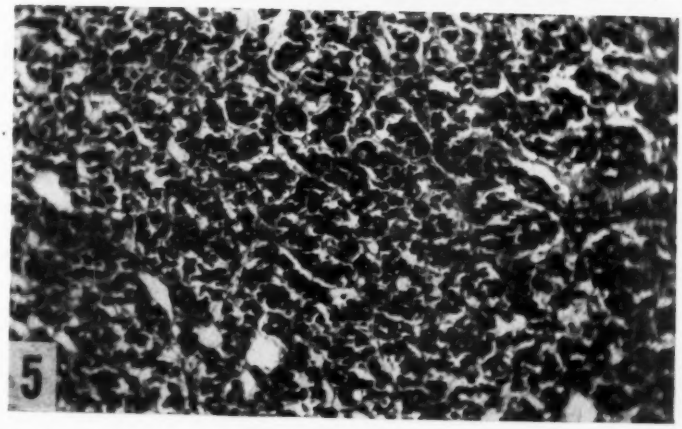
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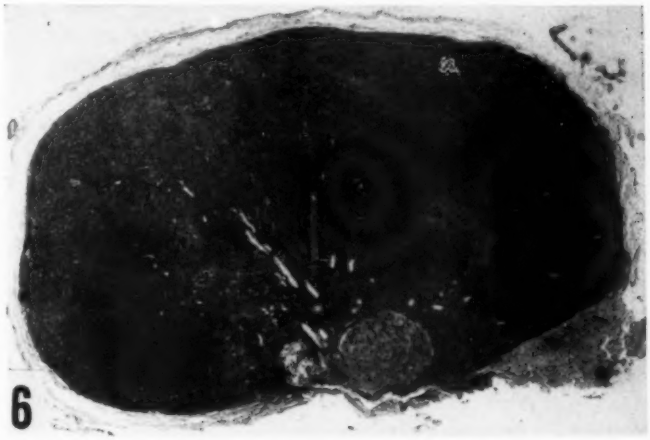
PLATE 105

FIGURE 6.—Pituitary gland in Case 3, 4½ months following delivery of 10,049 r tissue dose. The right lateral portion of the gland shows vascular scarring. Only a small segment of the posterior lobe is visible at this level. Hematoxylin and eosin stain. × 10.

FIGURE 7.—Higher magnification, demonstrating the fibrosed area in greater detail. The focal distribution is evident. Hematoxylin and eosin stain. × 43.

FIGURE 8.—Cellular detail of the adenohypophysis near the fibrosed area. The individual cells are normal, with abundant granules and delicate stroma. Hematoxylin and eosin stain. × 100.



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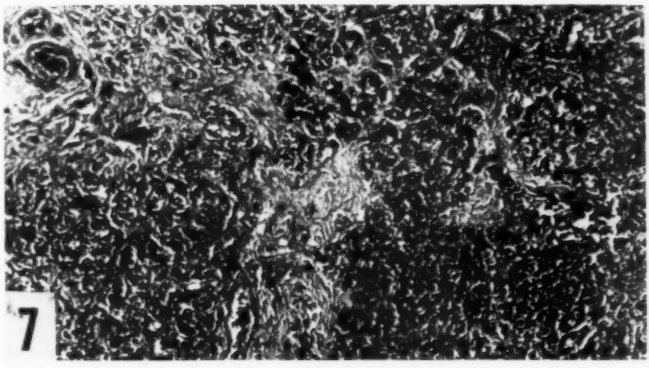

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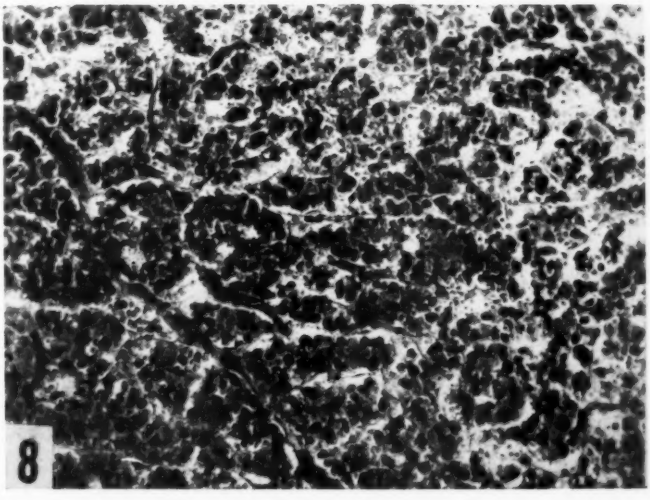

8

PRODUCTION OF TUMORS BY METHYLCHOLANTHRENE AND FETAL SKIN TRANSPLANTED TO DIFFERENT SITES IN MICE [1] [2]

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The observations of various investigators in the field of tumorigenesis show that the tissues of animals are not equally responsive to the action of such potent hydrocarbon carcinogens as 3,4-benzpyrene, 1,2,5,6-dibenzanthracene, or 20-methylcholanthrene (*1*). It is generally agreed that the skin, lungs, and subcutaneous tissues of mice are susceptible, whereas the testis, spleen, and liver are relatively refractory to the induction of tumors by the above carcinogens. Rous and Smith (*2*, *3*) observed that intramuscular transplantation of fetal skin together with methylcholanthrene in the mouse gave rise to neoplasms of the implanted tissue. Greene (*4*) published a note on his experiments in which mouse and guinea pig embryonic tissues impregnated with methylcholanthrene were transplanted homologously to different sites. Both carcinomas and sarcomas were induced with best results following subcutaneous implantations. Would neoplasms develop if transplants of mouse fetal skin were introduced into resistant sites such as the liver, spleen, or testis? Experiments reported in this paper show that they do.

MATERIALS AND METHODS

Strain C male mice (designated B alb C)[3] 2 to 4 months of age were employed as hosts. They were maintained in plastic cages, measuring 27.5 by 17.5 by 12.5 cm., generally 8 to a cage, and were fed pellets of Purina laboratory chow. In addition, they had access to tap water at all times.

All mice were implanted with fragments of fetal epidermis prepared as follows: Pregnant strain C females were killed by cervical dislocation 18 days following the discovery of a vaginal plug. Fetuses were removed aseptically, rinsed in several changes of sterile physiological salt solution, and placed in sterile petri dishes. A moist atmosphere was maintained by the introduction of about 2 cc. of saline at one side of each dish. Fetal skins were removed and placed together in another moist chamber. The

[1] Received for publication December 27, 1950.
[2] The co-operation and technical assistance of Mr. Frank M. McCathran is gratefully acknowledged.
[3] Law, L. W., J. Hered., 39 : 300–308, 1948.

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tissues were minced with fine scissors until resultant fragments approximated 1 mm. in diameter.

20-methylcholanthrene[4] was used either as crystals or as a 1-percent solution in olive oil, U. S. P. grade. In the former instance an attempt was made to enclose a few crystals within 0.04 to 0.05 cc. of minced skin which was then implanted with a 15 or a 12 gauge short-beveled trocar. Where the methylcholanthrene was introduced in an oily solution the procedure was adapted from one previously employed by Rous and Smith (*2*). Each mouse was injected once with 0.03 cc. of the olive-oil solution, 0.08 cc. of Locke's solution, and 0.04 to 0.05 cc. of minced skin. The above materials in the order named were drawn into and layered in a ¼-cc. tuberculin syringe. To facilitate the passage of materials, the tip of the syringe was bored out to a diameter of 1.5 mm. Upon injection, it was expected that each oil droplet would rise through the Locke's solution and toward the tissue. Often, however, part of the oil adhered to the plunger or to the barrel. This difficulty could be eliminated by the introduction of an air bubble behind the Locke's solution. With the syringe in an upright position, the air rose and separated the oil from the plunger. When the syringe was inverted, the bubble moved toward the tip, drawing the oil with it.

Materials were implanted by the usual methods in the subcutaneous tissues of the ear, axillary, and inguinal regions, and in the muscles of a hind limb. Transplantations to the liver, testis, and spleen were performed after the corresponding organs were exposed. Controls were injected similarly but without the carcinogen.

Mice in poor physical condition or those bearing subcutaneous or intramuscular tumors approximately 2 cm. in diameter were sacrificed. In the liver and spleen series, the majority of tumors were found at the time of autopsy. Tissues were fixed in Tellyesniczky's fluid, dehydrated, embedded in paraffin, sectioned at 6 to 8 micra, and stained with hematoxylin and eosin. Tumor diagnoses were based upon histologic examination of one slide for each tumor.[5] Semiserial sections were not prepared.

EXPERIMENTAL PROCEDURES AND RESULTS

The injected mice were divided into six series according to the site of implantation of the fetal epidermis. The results are summarized in table 1.

In Series A, transplants containing crystals of methylcholanthrene were implanted subcutaneously in the ear. Control animals received similar implants but without carcinogen. Two fibrosarcomas arose in the methylcholanthrene-treated mice. In two other instances, implant epithelium showed much hyperplasia with extensive downgrowth but no invasion or infiltration. No hyperplastic areas or tumors occurred in the controls.

[4] Obtained from Edcan Laboratories, South Norwalk, Conn., MP 179°—180° C.

[5] The author wishes to thank Dr. Thelma B. Dunn of the Pathology Section of the National Cancer Institute for her assistance.

TABLE 1.—*Results of experiments in which a mixture of strain C fetal skin and methylcholanthrene was implanted into strain C male mice*

Series	Mice used	Implantation site	Mode of carcinogen administration	Mice with tumors	Tumor diagnosis				Average tumor age	Average age at death, all mice
					fibrosarcoma	squamous-cell carcinoma	papilloma	"mixed"[1]		
	Number			*Number*	*Number*	*Number*	*Number*	*Number*	*Days*	*Days*
A experimental	19	Subcutaneous	Crystals	2	2				214	261
A control	20	In ear		0						314
B experimental	32	Subcutaneous in axillary and inguinal regions.	Olive oil solution	28	20	6	1	1	109	167
B control	20			0						266
C experimental	32	Intramuscular, hind limb	Olive oil solution	27	17	9		1	112	151
C control	20			0						332
D experimental	26	Intratesticular	Crystals	6	2	4			250	297
D control	10			0						336
E experimental	20	Intrahepatic	Olive oil solution	8	0	8			183	258
E control	22			0						290
F experimental	10	Intrasplenic	Crystals	8	4	3		1	158	175
F control	10			0						250
F experimental	10		Olive oil solution	6	0	5		1	150	186
F control	10			0						265

[1] Composed of both fibrosarcoma and squamous-cell carcinoma.

In Series B, transplants together with the solution of methylcholanthrene were placed in the axillary or inguinal regions. Tumors were induced in 28 mice and included fibrosarcomas, squamous-cell carcinomas, a papilloma (fig. 1), and a mixed tumor that contained elements of fibrosarcoma and squamous-cell carcinoma. No tumors were observed in the controls.

Mice of Series C received intramuscular implants together with the solution of methylcholanthrene. Twenty-seven of the mice bore tumors that were diagnosed as fibrosarcomas, squamos-cell carcinomas, and 1 mixed tumor. A lung metastasis was observed in a mouse bearing a squamous-cell carcinoma (fig. 2). None of the controls developed a tumor.

Implants exposed to crystalline methylcholanthrene were placed in the testes of mice of Series D. Six mice developed tumors of which two were fibrosarcomas and four were squamous-cell carcinomas (fig. 3). Tumors did not develop in the control mice.

In Series E, the liver parenchyma was utilized as a transplant site and a solution of the carcinogen was administered. Eight mice developed squamous-cell carcinomas in the liver (fig. 4.) No neoplasms were induced among the control mice.

In Series F, implants were placed in the spleen and exposed to either crystalline or dissolved methylcholanthrene. Eight tumors were induced in mice that had received the crystals and six in those that had received the solution. Four of the spleen tumors were diagnosed as fibrosarcomas, eight as squamous-cell carcinomas (fig. 5), and two as mixed tumors (fig. 6). None of the controls developed a tumor.

In view of the possibility that tumors induced from fetal skin might be more transplantable than those induced from adult skin, it was decided to test for such a difference. Thirty-five induced fibrosarcomas and 19 squamous-cell carcinomas were used in these tests. Each tumor was transplanted subcutaneously by trocar into 1 strain C male and 1 strain C3H male. Thirty-one of the sarcomas and 13 carcinomas grew in the strain C hosts. None grew in the strain C3H recipients. All animals were kept under observation for 30 days.

Histologically, the tumors induced from strain C fetal skin could not be distinguished from those obtained with adult skin.

DISCUSSION

In these experiments, a total of 149 mice received implants of a mixture of fetal epidermis and methylcholanthrene. Forty-five of these mice developed fibrosarcomas, 35 squamous-cell carcinomas, 1 papillomas, and 4 mixed tumors with sarcomatous and carcinomatous elements. Of the 85 tumors, 28 arose within the spleen, testis, and liver. These neoplasms developed despite the resistance of the mouse's spleen, testis, and liver to the carcinogenic action of hydrocarbons and provided an answer to the primary purpose of the experiment. The occurrence of tumors in these 3 sites would suggest that the skin implants were not influenced by those factors involved in the resistance of the surrounding host tissues.

Fetal skin encysted frequently upon transplantation to the liver and to other sites, especially when olive oil was included with the implants. Fibrosarcomas and squamous-cell carcinomas were produced in all sites implanted with fetal skin and methylcholanthrene except the liver. Here, only carcinomas were observed. It is possible, though unlikely, that the liver specifically inhibited the development of sarcomas. A greater possibility may be that, compared with other sites, methylcholanthrene exposed to liver tissues is removed or metabolized more rapidly. However, where this process was impeded, as by encystment, tumors arose in the tissue directly contacting the carcinogen. This could account for the skin carcinomas induced in the liver.

The latent period of tumorigenesis observed subcutaneously and intramuscularly could not be compared with that of the liver and spleen, since neoplasms were detected more readily and recorded sooner for the subcutaneous and intramuscular series. However, a carcinoma was observed in the liver of one mouse sacrificed on the 119th day and in the spleen of another sacrificed on the 77th day following implantation.

In the mouse, the liver and spleen appear to be relatively resistant to tumor metastasis. However, experiments of De Long and Coman (*5*) have shown that transplanted mouse tumors will grow in these two organs. It has been observed further, in these experiments, that primary tumors induced from fetal skin after transplantation to the liver and spleen will also continue to grow.

Some of the observed fibrosarcomas may have been derived from the action of methylcholanthrene upon host tissues. In these cases, their source of origin remained undetermined. On the other hand, there is no reason to assume that the induction of squamous-cell carcinomas in the liver as well as in other implantation sites was the result of the carcinogen acting upon host tissues.

Of 112 control mice that received implants of fetal skin alone, none developed a tumor (table 1). Rous and Smith (*2*, *3*) obtained similar results after 140 days. The present experiments extend these observations, since similar implants in mice maintained for as long as 390 days did not assume malignant properties in the absence of methylcholanthrene.

Histologically, tumors that originated in the fetal implants were indistinguishable from those derived from the corresponding adult tissue. This was not unexpected, for tissue differentiation is well advanced at 18 to 19 days of pregnancy when fetal skin was excised for implantation (*2*). In addition, these implants continued to differentiate after transplantation.

Despite a morphologic similarity between tumors induced with fetal as opposed to adult tissues, it was decided to test for differences in transplantability. It was found that the tumors derived from fetal skin implants in these experiments were not able to overcome strain barriers.

SUMMARY

Strain C fetal skin and methylcholanthrene transplanted subcutaneously, intramuscularly, and to the liver, spleen, and testis gave rise to neoplasms. These included fibrosarcomas, squamous-cell carcinomas, papillomas, and mixed tumors. Both sarcomatous and carcinomatous elements were observed in all sites but the liver, where only squamous-cell carcinomas were noted. Thus those factors responsible for the native resistance of liver, spleen, and testis to the hydrocarbon carcinogens were not observed to inhibit the formation of tumors in introduced fetal skin.

Transplantation of tumors derived from fetal skin in these experiments disclosed that the resultant neoplasms, in common with many tumors derived from adult tissues, were not able to overcome strain barriers.

REFERENCES

(*1*) HARTWELL, J. L.: Survey of Compounds Which Have Been Tested for Carcinogenic Activity, U. S. Public Health Service, Bethesda, Md., 371 pp. 1941.

(*2*) ROUS, P., and SMITH, W. E.: The neoplastic potentialities of mouse embryo tissues. I. The findings with skin of C strain embryos transplanted to adult animals. J. Exper. Med. 81: 597–620, 1945.

(*3*) SMITH, W. E., and ROUS, P: The neoplastic potentialities of mouse embryo tissues. II. Contributory experiments; results with the skin of C3H and Webster-Swiss embryos; general considerations. J. Exper. Med. 81: 621–646, 1945.

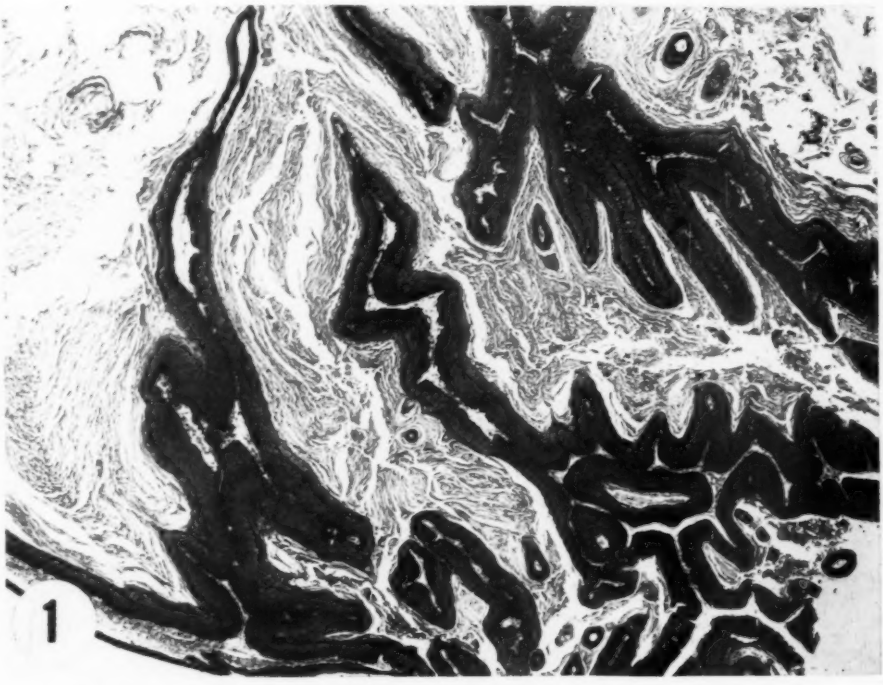
(*4*) GREENE, H. S. N.: The production of carcinoma and sarcoma in transplanted embryonic tissues. Science 101: 644–645, 1945.

(*5*) DE LONG, R. P., and COMAN, D. R.: Relative susceptibility of various organs to tumor transplantation. Cancer Research 10: 513–515, 1950.

PLATE 106

FIGURE 1.—Papillomas in an epidermal cyst induced in a subcutaneous transplant of fetal skin. Hematoxylin and eosin. × 25.

FIGURE 2.—Lung metastasis from a squamous-cell carcinoma induced in an intramuscular transplant of fetal skin. Hematoxylin and eosin. × 125.



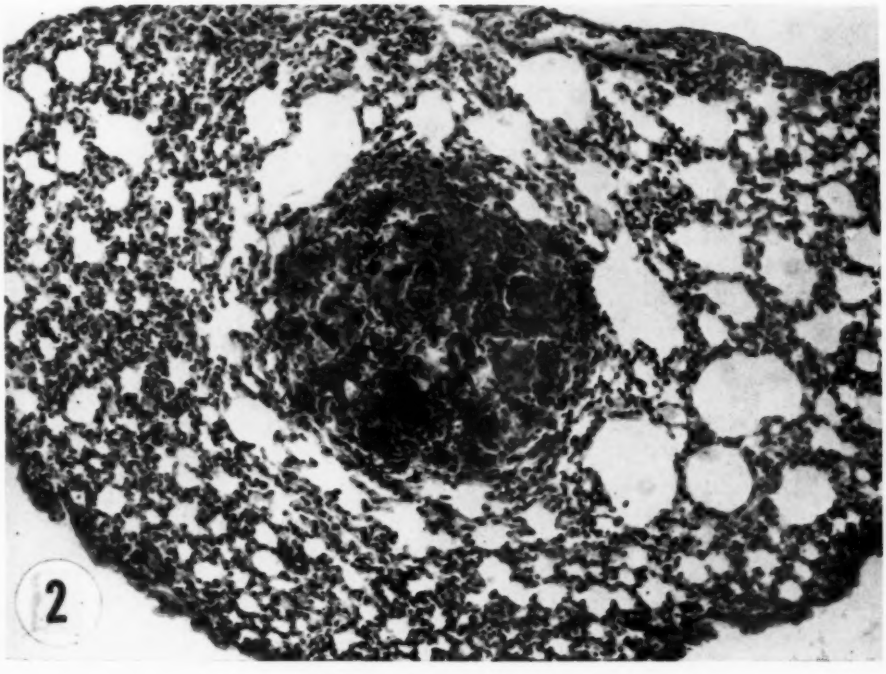


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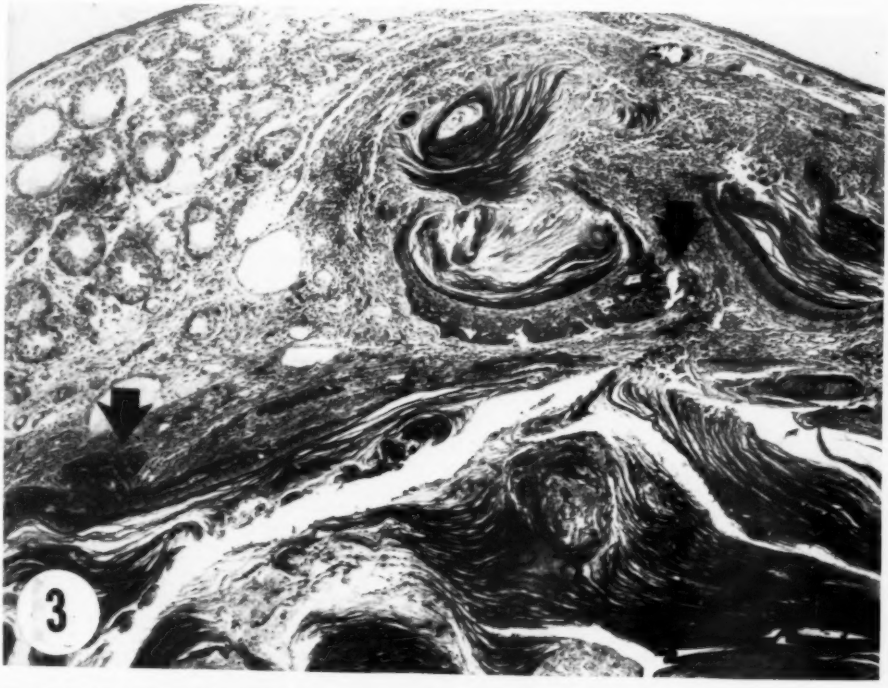
PLATE 107

FIGURE 3.—Squamous-cell carcinoma in the testis following implantation of fetal skin and methylcholanthrene. Infiltration of carcinoma cells marked by arrows. Hematoxylin and eosin. × 55.

FIGURE 4.—Squamous-cell carcinoma in the liver induced from fetal skin exposed to methylcholanthrene. Note bile-duct proliferation with great increase in epidermal thickness of duct walls within the tumor. Hematoxylin and eosin. × 85.



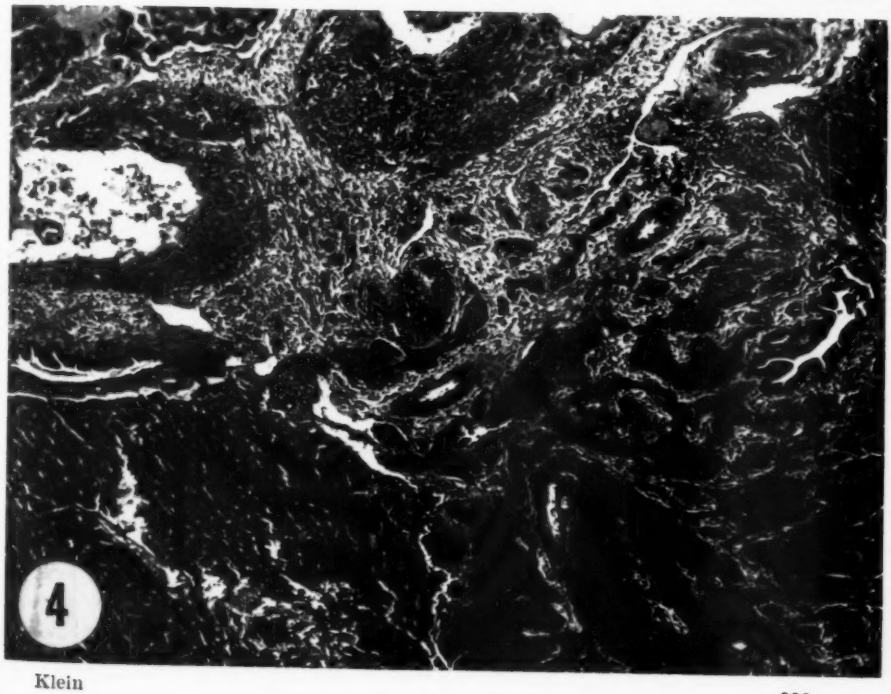


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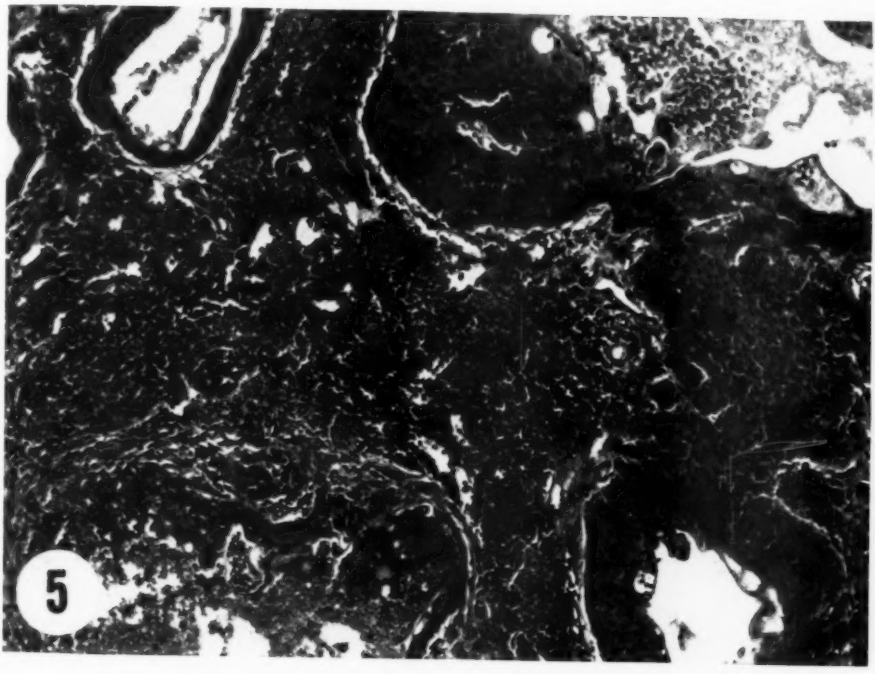
PLATE 108

FIGURE 5.—Squamous-cell carcinoma in the spleen following implantation of fetal skin and methylcholanthrene. Hematoxylin and eosin. × 125.

FIGURE 6.—"Mixed tumor" in the spleen following implantation of fetal skin and methylcholanthrene. The tumor contains merging elements of both sarcoma and squamous-cell carcinoma. Hematoxylin and eosin. × 300.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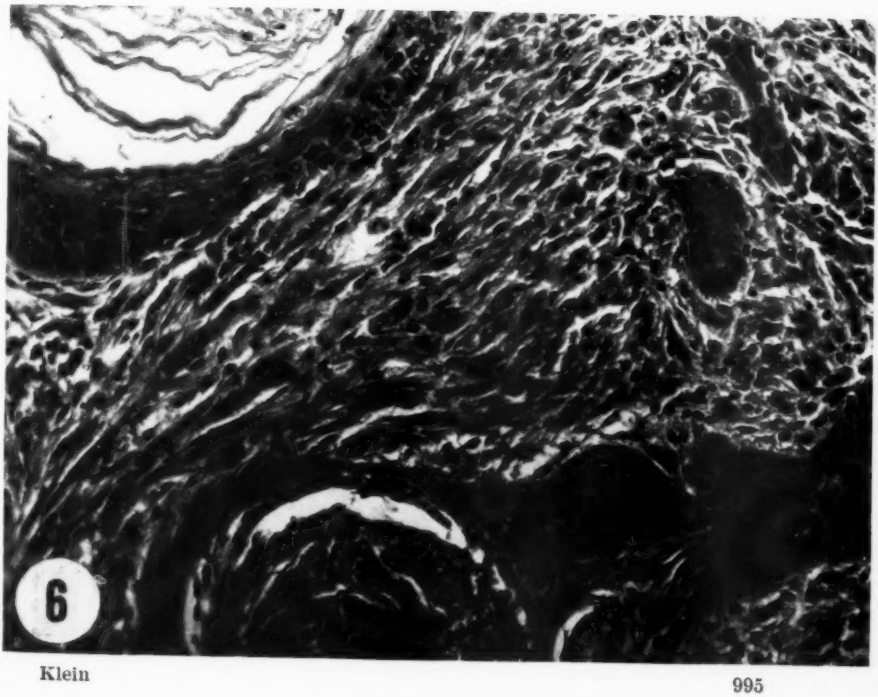
6

THE EFFECT OF INJECTIONS OF TUMOR FRACTIONS ON LIVER CATALASE ACTIVITY OF MICE[1,2]

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It is well established that the liver catalase activity of tumor-bearing rats and mice is significantly lower than that of normal animals (*2–9*). Although the cause of the lowered liver catalase is as yet unknown, the studies of Greenstein and his associates (*2–6*, *10*, *11*) on the liver catalase activity of tumor-bearing animals have shown that:

1. The decrease of liver catalase is progressive with the growth of the tumor and is most marked in animals bearing rapidly growing tumors.

2. The effect is reversible, *i. e.*, the liver catalase activity returns to normal after excision or spontaneous regression of the tumor. The liver catalase activity of rats from which a transplanted hepatoma had been removed, returned to normal within 24 to 48 hrs.

3. The fall of catalase activity in the liver is probably associated with a decreased catalase concentration.

4. Of the liver enzymes studied in tumor-bearing rats, only arginase showed any decrease in activity. The reduction in activity of this enzyme was much less striking than that of catalase.

5. Liver catalase activity is depressed much more than is kidney catalase except in the case of spontaneous mammary tumor in the mouse.

6. No reduction of liver catalase activity was observed in pregnant animals or in animals bearing implants of embryonic tissue.

Two alternative hypotheses have been suggested (*3*) to explain these facts: 1) that the tumor inhibits catalase by removing from the blood a component necessary for the normal functioning of this enzyme, or 2) that the tumor inhibits catalase by releasing a toxic substance or substances into the blood stream.

Attempts have been made to influence the liver catalase of normal animals by diet. Greenstein (*5*) found that a 3-day fast had no effect on liver catalase activity of rats. Weil-Malherbe (*7*) reported that the catalase activity of normal rat liver was not significantly changed by a high (45 percent casein) or a low (8 percent casein) protein diet, nor by daily intraperitoneal peptone injections. Intraperitoneal injections of

[1] Received for publication January 2, 1951.

[2] A preliminary report of this work has appeared (*1*).

sheep serum, however, raised the catalase activity of the liver. Miller (*12*) observed that in rats fasted for 7 days, the decrease in liver catalase activity paralleled or exceeded the general protein loss. Similar results were reported by Appelman *et al.* (*8*).

In an attempt to test the second hypothesis, Greenstein injected saline extracts of transplanted hepatoma 31 (including necrotic areas) intraperitoneally into normal rats and found that the liver catalase activity was not affected. However, in 1948, Nakahara and Fukuoka (*13*) reported the isolation of a fraction of certain human tumors which when injected into mice reduced the liver catalase activity within 24 hours. They were unable to isolate a similar material from normal tissue. Although there was considerable variation in the inhibitory effect among different tumor preparations, these workers postulated the existence in tumors of an agent capable of reducing liver catalase activity (*see* Addendum).

In the present investigation, various fractions of certain experimental tumors have been prepared and tested for their ability to lower liver catalase activity when injected intraperitoneally into C3H mice. Although a slight reduction in activity was obtained with material prepared by the method of the Japanese workers, a considerably more active agent was obtained by alcohol fractionation. Inbred strains and stable transplantable tumors were employed in an attempt to reduce variability.

EXPERIMENTAL PROCEDURE

The following tumors were employed: 1) C3HBA transplanted mammary tumor carried in C3H mice,[3] 2) Walker rat sarcoma,[4] and 3) AXCMCA2 transplanted fibrosarcoma carried in Dunning-Curtis rats. The mammary tumors were harvested 3 to 4 weeks after transplantation. Although the average tumor weight was from 4 to 5 gm., these tumors showed little or no necrosis grossly or microscopically. The AXCMCA2 transplanted fibrosarcoma was carefully dissected free of necrotic areas.

Isolation.—Acetone powders were prepared by blending the tumor with 3 volumes of cold acetone in a Waring blender at 5° C. The material was filtered, resuspended in 2 volumes of acetone, refiltered, washed with ether and dried *in vacuo* at 5° C.

An aqueous suspension of the acetone powder (1 gm. of powder to 40 cc. of water) was refluxed for 3 hours on a boiling water bath with continuous stirring. (Fresh tumor blended for 15 minutes with 4 volumes of water in a Waring blender at 5° C. was occasionally used instead of the acetone powder suspension. However, with tumors containing much lipid material, poor separations were subsequently obtained by alcohol fractionation.) The suspension was centrifuged for 30 minutes in the Sharples centrifuge at 62,000×g. The precipitate was discarded, the supernate

[3] The transplanted mammary tumor and fibrosarcoma were kindly supplied by Dr. M. K. Barrett.

[4] The authors wish to thank Dr. G. B. Mider for donating a generous supply of lyophilized necrotic and nonnecrotic Walker sarcoma tissue.

evaporated *in vacuo* to one-tenth of its original volume and centrifuged again in the Sharples for 30 minutes. The slightly opalescent, yellow supernatant was chilled to 0° C. and cold ethanol was added to a final concentration of 10 percent. After standing three hours at 0° C., the solution was centrifuged for 30 minutes in the Sharples centrifuge. The precipitate was washed with absolute ethanol and ether and dried. The fractionation was continued by successively increasing the alcohol concentration to 60 percent. The pH was adjusted to 4.5 for the final alcohol precipitation. Each fraction was dissolved in water (60 cc. per gm.), adjusted to pH 7.4, and the insoluble material was removed by centrifugation to yield a clear yellow solution. Precipitation was carried out at room temperature by adding glacial acetic acid to pH 3.5 and 2 volumes of ethanol. This precipitate was centrifuged and washed with absolute ethanol and ether. The fractions thus obtained were nearly white powders which when stored *in vacuo* at 5° C. retained their activity for many months.

Active material was also obtained by means of a more convenient procedure. The acetone powder was dissolved in water, refluxed, and centrifuged as described above. The supernate was evaporated to one-fourth its original volume and brought to a pH of 7.4. Absolute ethanol was added at 0° C. to a final concentration of 10 percent. The insoluble material was centrifuged and discarded. The solution was adjusted to pH 5 by addition of glacial acetic acid and the ethanol concentration increased to 20 percent. A voluminous white precipitate was obtained, which was centrifuged and washed with absolute ethanol and ether. The powder was dissolved in water and precipitated as described above. The yield of active material was 80 mg. per gm. of acetone powder.

Assay.—The liver catalase activity of normal C3H mice (2 to 5 months old) was determined 24 hours following intraperitoneal injection of a neutral solution of the material to be tested. In any given assay, the injected mice and the controls (injected with 0.85 percent sodium chloride) were of the same age and sex. Two or more mice were employed for each assay except in a few cases in which insufficient material was available. The animals were killed by decapitation and bled. The livers were quickly removed, suspended in 10 volumes of cold M/15 phosphate buffer of pH 6.8, and homogenized in a Potter-Elvehjem homogenizer for 1 minute at 1,250 r. p. m. in an ice bath. The homogenates were diluted with 10 volumes of buffer immediately prior to the determination of catalase activity. The volumetric procedure described by Greenstein (*2*), in which the time in seconds was measured for the evolution of 10 and 20 cc. of oxygen from a 29 percent hydrogen peroxide solution, was used. Since the activity expressed as percent of control value agreed at the two levels, only values for evolution of 10 cc. of oxygen are reported. When sodium perborate was used as the substrate (*14*), the homogenates were diluted with 100 or 200 volumes of buffer. The nitrogen content of homogenates prepared from experimental and control animals was not significantly different.

RESULTS

Inhibitory Effect of Various Fractions of Normal and Neoplastic Mouse Tissues

An acetone powder of the C3HBA mammary tumor was fractionated as described above. Each fraction was tested in 100-mg. doses, and the results of these assays are given in table 1. The liver catalase activity of animals

Table 1.—*Effect of fractions of mouse mammary tumor, liver, and muscle on liver catalase activity*

Ethanol fraction	Tumor (preparation 1)		Muscle		Liver	
Percent	Weight [1]	Activity [2]	Weight [1]	Activity [2]	Weight [1]	Activity [2]
0–10	.577	67	0.232	94	0.049	
10–15	2.230	42	.183	67		
15–20	1.465	44	.805	83	.244	67
20–30	.154	43	.360	56	.572	67
30–50	.180	48	.125	100	.417	67
50–60	1.760	56	.790	110	.374	110
60; pH 4.5	1.220	67				
Total weight	7.586		2.495		1.656	

[1] Calculated on the basis of 100 gm. of acetone powder.

[2] Percent of average control value (1.8 cc O_2 per second). One cubic centimeter of 1/100 liver homogenate was treated with 8 cc. of M/15 phosphate buffer at pH 6.8, and 1 cc. of 29 percent hydrogen peroxide at 25° C.

injected with fractions precipitated by 15 to 50 percent ethanol was less than half of that of the control animals. A total of 7.586 gm. of material was obtained per 100 gm. of acetone powder, and of this, 5.789 gm. exhibited good activity. A number of subsequent fractionations were carried out with similar results.

The loss of liver catalase activity obtained by injecting fractions of the C3HBA mammary tumor is of the same order of magnitude as that found in animals bearing this tumor. The liver catalase activity of the tumor-bearing animals used in this study was 37 to 50 percent of the average value obtained for normal C3H mice.

When acetone powder obtained from muscle of normal C3H mice was fractionated (table 1), the fractions precipitated between 10–15 and between 20–30 percent ethanol lowered liver catalase activity to a moderate extent. However, fractionation yielded a total of 2.495 gm. of material, of which only 0.543 gm. exhibited inhibitory activity. There was therefore considerably less active material obtained from normal muscle than from the tumor, by the fractionation procedure employed. Similar results were obtained with liver.

A number of assays were performed employing sodium perborate as the substrate rather than hydrogen peroxide. The values expressed as percent of the control value were in close agreement with determinations performed by the manometric hydrogen-peroxide procedure.

Injection of 0.85 percent sodium chloride, desoxyribonucleic acid, ribonucleic acid, and crystalline bovine serum albumin had no effect on

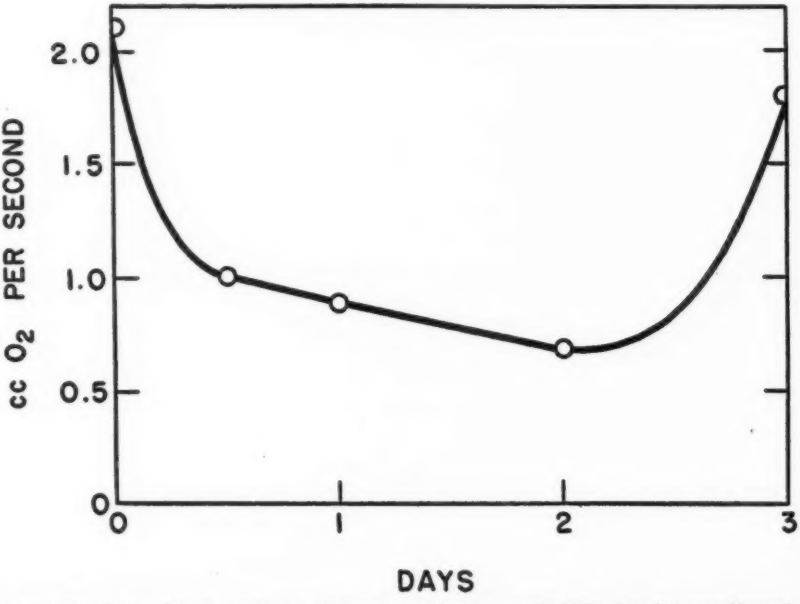
TABLE 2.—*Effect of various materials on liver catalase*

Material	Dose	Catalase Activity [1]
Mammary tumor:	(*mg.*)	*percent*
Preparation No. 2 [3]	50	[2] 63
Preparation No. 2 [3]	100	[2] 42
Preparation No. 3 [4]	50	56
Preparation No. 3 [4]	100	20
Preparation No. 4 [5]	100	48
Crystalline bovine serum albumin	100	97

[1] Expressed as percent of control value.
[2] Sodium perborate assay procedure.
[3] 20–40 percent ethanol fraction.
[4] 15–20 percent ethanol fraction.
[5] 20–30 percent ethanol fraction.

liver catalase. In contrast, active material was repeatedly obtained from the mouse mammary tumor (table 2). The lowered liver catalase activity produced by injection of the active tumor fraction was observed 12 hours after injection. The effect was present 24 and 48 hours after administration of the active fraction, and a normal level of liver catalase was again established after 72 hours (text-fig. 1).

Fractions of mouse mammary tumor that were effective in lowering the liver catalase to about 50 percent of the control level when injected into normal C3H mice were tested *in vitro*. Mouse liver homogenates were incubated with active tumor fractions for 30 and 60 minutes at 37° C. The



TEXT-FIGURE 1.—Liver catalase activity at various intervals following intraperitoneal injection of 100 mg. of active tumor fraction.

mixtures (containing between 0.1 and 10 mg. of tumor fraction per cc.) were then diluted and tested for catalase activity. The activity values in the presence and absence of tumor fractions were identical.

Effect of Injection of Mammary Tumor Fractions on Certain Other Liver Enzymes

It was of interest to determine whether injections of tumor fractions affected the activity of other liver enzymes. Five mice were given a single dose of 100 mg. of an active mammary tumor fraction. The activity of several enzyme systems was determined 24 hours after injection (table 3). Although the liver catalase values were 56 percent of the average

Table 3.—*Effect of injection of mammary tumor fraction on certain enzymatic activities of mouse liver*

	Catalase activity [1]	Transaminase [2]	Dehydropeptidase [3]	Lactic dehydrogenase [4]
Injected with tumor fraction	1.1	4.19	-------	223
	1.1	3.78	15.5	300
	.96	3.56	15.8	231
	.85	3.19	16.6	270
	1.2	3.86	15.5	270
Average	1.0	3.71	15.9	259
Injected with 0.85 percent NaCl	1.7	4.12	17.5	231
	1.7	4.12	17.6	223
	1.8	4.27	17.2	194
	1.9	4.61	16.0	224
Average	1.8	4.28	17.1	218

[1] Cubic centimeters of oxygen released per second.

[2] Glutamic acid disappearance (μM) in the presence of pyruvate in 15 minutes. Reaction mixtures contained 1 cc. of homogenate (1:50), 10 μM glutamic acid, 20 μM Na pyruvate, and veronal buffer at pH 7.2 in a final volume of 4 cc. Glutamic acid was determined by enzymatic decarboxylation.

[3] μM NH_3 formed from glycyldehydroalanine by 1 cc of 1: 10 homogenate in 20 minutes (*15*).

[4] μM pyruvate reduced per minute per gram of liver at 26° C. (*16*).

control value, no significant changes occurred in transaminase, dehydropeptidase, or lactic dehydrogenase activity. It may therefore be concluded that injection of the tumor fraction did not produce a general depression of enzymatic activity.

Effect of Fractions of Normal and Neoplastic Rat Tissues

The effect of fractions obtained from Walker sarcoma and rat fibrosarcoma on the liver catalase of C3H mice is somewhat lower than that observed with mouse mammary tumor fractions (table 4). It is of interest that the values for fractions obtained from the necrotic and nonnecrotic areas of the Walker tumor produced effects of about the same order of magnitude. However, the necrotic areas were toxic and killed the animals injected with the 20–30 and 50–60 percent ethanol fractions. Very low yields of material were obtained by fractionation of normal rat spleen and intestine. Only a few fractions were large enough to permit assay. The fractions obtained from spleen by precipitation with

TABLE 4.—*Effect of certain normal and neoplastic rat tissue fractions*

Ethanol fraction	Walker sarcoma				Fibrosarcoma		Liver	
	Nonnecrotic		Necrotic					
Percent	Weight [1]	Activity [2]	Weight [1]	Activity [2]	Weight [1]	Activity [2]	Weight [1]	Activity [2]
0–5	0. 057	[4] 110	1. 941	55	0. 553	64	0. 027	------
5–10	1. 239	79						
10–15	. 258	58	3. 584	55	1. 172	68	. 047	------
15–20	1. 129	68	. 919	60	1. 235	73	. 129	86
20–30	2. 011	58	. 379	[3]	. 414	64	. 124	110
30–50	1. 317	63	1. 006	65	. 312	64	. 440	86
50–60	. 381	68	. 508	[3]	0	------	0	------
Total	6. 392	------	8. 337	------	3. 686	------	. 767	------

[1] Calculated on the basis of 100 gm. of acetone powder.
[2] Percent of average control value (1.9 cc. O_2 per second).
[3] Animal died.
[4] 50 mg. of material injected.

10–15 and 15–25 percent ethanol and those obtained from intestine by precipitation with 30–50 and 50–60 percent alcohol were inactive. The fractions obtained from rat liver that were large enough for assay were also found to be inactive.

CERTAIN PROPERTIES OF ACTIVE TUMOR FRACTIONS

Dialysis of active fractions against water for 36 hours had no effect on the activity. This finding as well as preliminary ultracentrifugal studies suggested that the active agent might possess itself or be associated with particles with a molecular weight of at least 40,000. However, it was subsequently found that digestion of active fractions with 6 N hydrochloric acid at 100° C. for 12 to 48 hours did not affect the activity (table 5). Furthermore when the digested material was dialyzed, the activity was

TABLE 5.—*Effect of acid digestion on activity* [1]

Fraction	Duration of digestion (hours)	Catalase activity (percent of control)
Mammary tumor preparation No. 4:		
Fraction 10–20	0	50
" "	18	58
Fraction 10–20 [2]	0	46
" " [2]	18	48
" " [2]	48	39
Fraction 20–30	0	59
" "	12	63
Walker sarcoma, nonnecrotic, Fraction 15–20	0	64
" " " " " "	12	69
Amino acid mixture [3]	0	91

[1] All assays were carried out with 100-mg. doses.
[2] Precipitated at pH 4.5.
[3] A mixture of crystalline optically-pure L-amino acids containing alanine, glycine, serine, proline, aspartic acid, arginine, valine, threonine, phenylalanine, hydroxyproline, α-aminobutyric acid, and isoleucine in a concentration of 5 percent, tryptophan in a concentration of 2 percent, lysine in a concentration of 8 percent, leucine in a concentration of 10 percent, and glutamic acid in a concentration of 20 percent was injected in doses of 100 mg.

found to be dialyzable. Digestion of an inactive fraction obtained from rat intestine did not significantly change the activity. Intraperitoneal injection of a mixture of the amino acids present [8] in the digested material in C3H mice did not affect the liver catalase activity.

Although all of the fractions obtained by alcohol precipitation exhibited a maximum absorption in the utraviolet at 2,600 Å, there was no correlation between activity and absorption at this wave length.

DISCUSSION

The present findings are compatible with the hypothesis that the lowered liver catalase of tumor-bearing animals is due to an inhibitory substance elaborated by the tumor. The inhibition resulting from injection of mammary tumor fractions is of the same order of magnitude as that found in livers of mice bearing this tumor. The fractions obtained from the normal tissues studied possessed very little activity as compared with material derived by the same procedure from mammary tumor. The liver catalase activity returned to normal levels 72 hours after injection of active fractions. It is of interest that a 48-hour recovery period was found for the liver catalase activity of rats following extirpation of hepatomas (*5*). Another similarity between the effects of injection of tumor fractions and the findings with tumor-bearing animals is the absence of a general depression of enzyme activity. The present results are in general agreement with those of Nakahara (*13, 17, 18*) on human tumors.

It would appear that the active material is effective in both a nondialyzable and a dialyzable form, and that it is very stable to heat and strong acid. Further studies on the chemical nature of the active agent are in progress.

SUMMARY

1. Certain tumors and normal tissues were subjected to ethanol fractionation, and these fractions were injected intraperitoneally into C3H mice. The liver catalase activity of these mice and control animals was determined.

2. Fractions obtained from C3HBA mammary tumor lowered the liver catalase activity by more than 50 percent within 24 hours following a single intraperitoneal injection.

3. The effect on the liver catalase activity was observed 12 hours after injection, and after 72 hours the liver catalase activity was within normal limits.

4. Fractions obtained from normal tissues possessed considerably less ability to lower liver catalase.

5. The depression of liver catalase activity was not accompanied by a significant decrease in liver lactic dehydrogenase, dehydropeptidase, and glutamic-alanine transaminase.

6. The activity of tumor fractions was not affected by digestion with 6 N hydrochloric acid for 48 hours.

[8] The amino acids present in the digest were identified by paper chromatographic procedures by Dr. H. A. Sober.

7. The present findings are compatible with the hypothesis that the lowered liver catalase activity of tumor-bearing animals is due to a compound (or compounds) elaborated by the tumor.

ADDENDUM

After this paper was submitted a very interesting report by D. H. Adams appeared (Brit. J. Cancer 4: 183, 1950). This author found that the liver catalase activity of mice injected subcutaneously with homogenized sarcoma (S37) decreased significantly 24 and 48 hours after injection, rose to normal by the 4th day, and diminished again during the growth of the new tumors. Injection of a variety of normal tissues, including whole embryo tissue, produced no significant change in liver catalase activity.

REFERENCES

(1) GREENFIELD, R. E., and MEISTER, A: Studies on the inhibition of liver catalase in tumor-bearing animals. Cancer Research 10: 222, 1950.

(2) GREENSTEIN, J. P.: Titration of the liver catalase activity of normal and tumor-bearing rats and mice. J. Nat. Cancer Inst. 2: 525–530, 1942.

(3) ———: Further studies of liver catalase activity of tumor-bearing animals. J. Nat. Cancer Inst. 3: 397–404, 1943.

(4) GREENSTEIN, J. P., and ANDERVONT, H. B.: Liver catalase activity of tumor-bearing mice and the effect of spontaneous regression and of removal of certain tumors. J. Nat. Cancer Inst 2: 345–355, 1942.

(5) GREENSTEIN, J. P., JENRETTE, W. V., and WHITE, J.: The liver catalase activity of tumor-bearing rats and the effect of extirpation of the tumors. J. Biol. Chem. 141: 327–328, 1941.

(6) ———: The liver catalase activity of tumor-bearing rats and the effect of extirpation of the tumors. J. Nat. Cancer Inst. 2: 283–291, 1941.

(7) WEIL-MALHERBE, W., and SCHADE, R.: Studies on the liver catalase of normal and cancerous rats. Biochem. J. 43: 118–125, 1948.

(8) APPLEMAN, D., SKAVINSKI, E. R., and STEIN, A. M.: Catalase studies on normal and cancerous rats. Cancer Research 10: 498–505, 1950.

(9) EULER, H. V., and HELLER, L.: Catalase activity in liver fractions of normal and sarcomatous rats. Ztschr. f Krebsforsch. 56: 393–406, 1949.

(10) GREENSTEIN, J. P., and ANDERVONT, H. B.: Liver catalase activity of pregnant mice and of mice bearing growing embryonic implants. J. Nat. Cancer Inst. 4: 283–284, 1943.

(11) GREENSTEIN, J. P., ANDERVONT, H. B., and THOMPSON, J. W.: Kidney and blood catalase activity of tumor-bearing animals. J. Nat. Cancer Inst. 2: 589–594, 1942.

(12) MILLER, L. L.: Changes in rat liver enzyme activity with acute inanition. J. Biol. Chem. 172: 113–121, 1948.

(13) NAKAHARA, W., and FUKUOKA, F.: A toxic cancer tissue constituent as evidenced by its effect on liver catalase activity. Japan. Med. J. 1: 271–277, 1948.

(14) FEINSTEIN, R. N.: Perborate as substrate in a new assay of catalase. J. Biol. Chem. 180: 1197–1202, 1949.

(15) GREENSTEIN, J. P., PRICE, V. E., and LEUTHARDT, F. M.: Studies on the possible multiple nature of dehydropeptidase I. J. Biol. Chem. 175: 953–962, 1948.

(16) MEISTER, A.: Lactic dehydrogenase activity of certain tumors and normal tissues. J. Nat. Cancer Inst. 10: 1263–1271, 1950.

(17) NAKAHARA, W., and FUKUOKA, F.: Toxohormone: A characteristic toxic substance produced by cancer tissue. "Gann" 40: 45–69, 1949.

(18) ———: Purification of toxic hormone. A second study on toxohormone, a characteristic toxic substance produced by cancer tissues. "Gann" 41: 47–55, 1950.

DIFFERENTIAL EFFECTS OF ROENTGEN RAYS ON CELL PERMEABILITY AND ON CELL CLEAVAGE. EXPERIMENTS WITH EGG CELLS OF *ARBACIA PUNCTULATA* [1] [2]

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Many radiobiologists believe that exposure of living cells to Roentgen rays, or to other forms of irradiation, leads to changes in permeability, and that to such changes in permeability may be attributed, in part at least, the injurious effects of ionizing irradiations on neoplastic as well as on normal cells. This concept, however, has been derived from experiments that have been mainly qualitative rather than quantitative, and in many instances the methods used, as well as the interpretation of the results, are open to criticism. The older literature has been reviewed by Heilbrunn and Mazia (*1*). In more recent years, although permeability determinations have become more quantitative, few such seem to have been made on irradiated cells. We have been able to find only 2 papers published during the past few years that are pertinent to the present experiments. Ting and Zirkle (*2*) studied permeability to water and certain nonelectrolytes, using beef erythrocytes exposed to 33,000 Roentgen units. They observed a 20 percent increase in permeability to water, but only a 6 percent increase to thiourea, and a 3.5 increase to ethylene glycol. Reed (*3*) exposed the unfertilized eggs of a sea urchin, *Strongylocentrotus purpuratus*, to ultraviolet light of various wavelengths (2,483 to 3,130 A) and found no significant effect on permeability to water, to ethylene glycol, or to certain other solutes.

The biological importance of ionizing irradiations is rapidly increasing. They are widely used in studying a great variety of normal and abnormal processes, including neoplasia. This fact makes it desirable to obtain precise information as to the mechanism of irradiation effects on cells.

The present paper deals with the action of Roentgen rays on permeability of unfertilized Arbacia eggs to water, to ethylene glycol, and to diethylene glycol. In particular, the more or less immediate rather than

[1] Received for publication January 12, 1951.

[2] This investigation has been aided by a grant from the Committee on the Advancement of Research of the University of Pennsylvania.

the late effects were studied following exposure of these cells to 100,000 r. With the same material the action of Roentgen rays on cell cleavage has been investigated and compared with the effect on permeability.

MATERIAL AND METHOD

The unfertilized egg cells of the sea urchin, *Arbacia punctulata*, were selected as test objects because, for the purpose of the present experiments, they have several advantages: large numbers of isolated cells can be obtained in an uninjured condition; they are spherical in shape and, in any one animal, of fairly uniform size. These characteristics permit quantitative measurements of permeability by the methods we wished to use. Also, the resting cells can be activated (fertilized) readily by adding normal sperm, whereupon normal cells undergo cleavage at a definite rate and in an almost constant pattern, while in injured cells the division rate may be delayed, the cleavage pattern may become atypical and the cells may disintegrate. Thus it is possible to determine whether the experimental procedures have altered cell cleavage and whether they have produced irreversible injury or death. Since exposure of Arbacia eggs to Roentgen rays is known to interfere with the orderly process of cleavage, it becomes important to correlate these effects with possible changes in permeability.

The technique for obtaining the eggs and the precautions necessary for keeping them in good condition during the course of the experiments have been described previously (*4*).

For irradiation, eggs from a single animal were first washed in filtered sea water and a portion of them placed in a flat-bottomed celluloid capsule, 7 cm. in diameter and 1.5 cm. in depth, care being taken to have the eggs distributed in a thin layer. The Roentgen-ray generator at the Marine Biological Laboratory consists of 2 tubes in alternate parallel cross-firing in center; it is operated at 182 kv, and 25 ma per tube, with an 0.15 mm. copper equivalent filter. The distance from the center of each target to the test object is 9.5 cm., and the rate of delivery 7,190 r per minute. The total amount of irradiation used was 100,000 roentgen units, measured in air, and delivered in approximately 14 minutes.[3]

After irradiation the cells were divided into several lots and placed in bowls of fresh sea water. To one lot normal sperm was added within 3 to 8 minutes after the termination of the exposure and at the same time control eggs were fertilized. Other lots of irradiated cells were used for measurement of permeability. The measurements of permeability were begun within 10 minutes after irradiation. Usually, the measurements were repeated 2 or 3 times within the next 1 or 2 hours with different lots of cells from the same animal.

Permeability values were obtained for water, for ethylene glycol, and for diethylene glycol. The values of permeability were computed from

[3] We wish to express our appreciation for the technical assistance of Mr. H. R. Hyde, Department of Experimental Radiology, Marine Biological Laboratory.

measurements of the volume changes that take place when the eggs are transferred from sea water, with which they are in osmotic balance, to a hypotonic or to a hypertonic medium. In most of the determinations, the course of osmotic swelling or shrinking was followed by means of a diffraction method, the details of which have been described (*4*, *5*). Briefly stated, the method is based upon the principle that a beam of parallel monochromatic light from a slit source is diffracted in passing through a homogeneous suspension of small particles. Each individual object contributes to the diffracted light; hence a single measurement of the diffraction pattern gives the average size of all the objects in suspension—in the present experiments many thousands of cells—thereby eliminating errors due to individual variation in size. As viewed through a telescope this diffraction pattern consists of alternate dark and light bands (minima and maxima of intensity) symmetrically arranged on either side of the image of the slit. The angular displacement of a given diffraction band from the central image of the slit is determined by setting the crosshairs of the telescope upon it, and reading on a scale the angle through which the telescope has been rotated. From such measurements the average size of the objects causing diffraction may be computed. Precise measurements can be made at intervals of a few seconds, a factor of importance when volume changes are rapid.

In order to check the results, a second (photoelectric) method was used in some experiments. This method is based upon the principle that the amount of light passing through a homogeneous suspension of semiopaque cells varies inversely with the volume of the cells. The rate of change in intensity of light during the course of change in cell volume is obtained in the form of galvanometric records (*6*).

RESULTS

The experimental results will be presented in the following order: effect of irradiation on size, shape, and tendency to cytolysis of the cells; on permeability to water during the course of swelling in hypotonic sea-water; on permeability to water during the course of shrinking in an initially hypertonic solution of a penetrating solute; on permeability to ethylene glycol, and to diethylene glycol; on the course of volume changes as recorded galvanometrically; on permeability values at different times after irradiation; and on the effect of irradiation on cell cleavage.

Size, Shape, and Tendency to Cytolysis

As a preliminary to permeability determinations it was necessary to find out whether exposure of the eggs would lead to changes in their initial size or shape, or in their tendency to cytolysis. A change in initial size, while not interfering with the experimental procedures, would have to be taken into account in the computations, but change in shape or an early tendency to cytolysis would prevent the use of the diffraction method for measuring the cells. Fortunately, none of these changes occurred

within several hours after irradiation. In table 1 are given measurements of 6 different lots of irradiated eggs and the corresponding controls. It is seen that irradiation did not induce change in the initial size of the eggs. The mean volumes of the control eggs was found to be $2{,}185 \times 10^2$ μ^3; after irradiation the mean volume was $2{,}176 \times 10^2 \mu^3$.

Similarly, no change in shape of the eggs was observed upon careful examination with the microscope. Nor was there any evidence of cytolysis during the first 2 or more hours. But, upon standing for several hours, approximately 5 percent of the irradiated cells had cytolyzed, indicating that they had been irreparably damaged by the Roentgen rays.

TABLE 1.—*Comparison of volume of control and of irradiated cells. All measurements were made within 2 hours after exposure to 100,000 r. The figures must be multiplied by 100 to obtain actual volumes in cubic micra. No significant difference occurs in the 2 columns of figures*

Lot No.	Original volume of control cells	Volume after irradiation	Lot No.	Original volume of control cells	Volume after irradiation
I	2, 253	2, 227	V	2, 442	2, 340
II	2, 088	2, 037	VI	2, 253	2, 253
III	2, 020	2, 148			
IV	2, 054	2, 054	Mean	2, 185	2, 176

PERMEABILITY OF THE CELLS TO WATER

The initial size of irradiated and nonirradiated eggs was obtained by measuring them in ordinary sea water, as in the example given above. To determine their permeability to water, 2 methods were used.

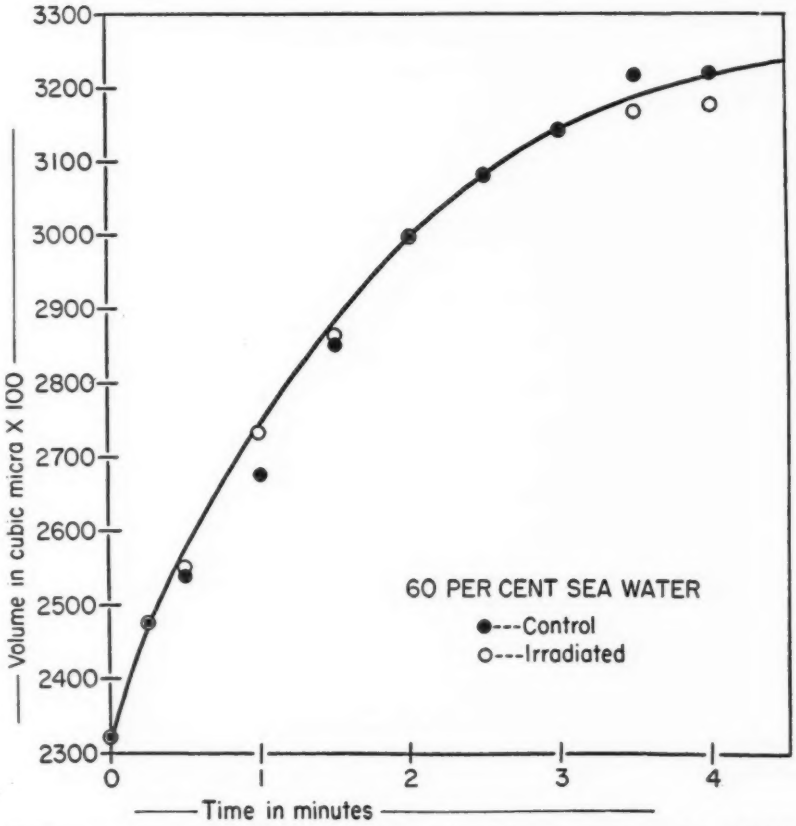
First method.—The measured cells were transferred to sea water diluted to 60 percent (occasionally to 70 or 50 percent).[4] In these hypotonic solutions water entered under the driving force of osmotic pressure, and the cells swelled. The course of swelling was followed by measuring the diffraction pattern at convenient intervals. From such measurements, the permeability of the cell to water was then computed from the equation:

$$K = \frac{dV}{dt} / S.(P - P_e)$$

where $\frac{dV}{dt}$ is the rate of change of cell volume due to penetration of water; S area of cell surface; $(P - P_e)$ the difference in osmotic pressure between the interior of cell and the medium; and K expresses the permeability of the cell to water. In the units used, K gives the number of cubic micra of water that enter the cell per minute through 1 square micron of surface per atmosphere of pressure.

The details of calculation have been given in previous papers (*5, 9, 10*). It is not necessary here to discuss the computation of permeability further, as the results of the experiment are illustrated in text-figure 1. The

[4] By 60-percent sea water is meant a solution consisting of 60 parts sea water and 40 parts distilled water. A suspension of egg cells in 60-percent sea water is conveniently prepared by mixing 2 parts of cell suspension in 100 percent sea water with 8 parts of 50-percent sea water, both solutions having first been brought to the desired temperature.



TEXT-FIGURE 1.—Comparison of the course of swelling of control and irradiated Arbacia eggs in 60 percent sea water. The curve is constructed by plotting volumes against time, the values representing the mean volumes of duplicate experiments. It is seen that the course of swelling is very similar for both control and irradiated cells, indicating absence of measurable effect of irradiation on permeability to water.

observed volumes of both irradiated and control cells during their course of swelling in 60 percent sea water have been plotted against corresponding times, and a smooth curve has been drawn through the points. It was found that the volumes of both kinds of cells were closely scattered about the curve. The value of K in the experiment was 0.18. In 7 additional experiments, of which 3 were done in triplicate, the values of K have ranged from 0.14 to 0.19.[5]

From these experiments it was concluded that Roentgen irradiation did not alter to a measurable degree the permeability to water of unfertilized Arbacia egg cells.

[5] In these computations, cell volumes have not been corrected for the amount of osmotically inactive substance, b; such correction slightly lowers the value of the permeability constants. Since the present experiments are concerned with comparative values, allowance for b was considered unnecessary.

Second method.—This method, developed by Jacobs (*11*), makes it possible to evaluate simultaneously cell permeability to water and to a penetrating compound. The cells are first measured in ordinary sea water and are then placed in a medium made by dissolving in sea water 0.5 M of a relatively harmless substance to which the cells are permeable: in the present experiments, ethylene glycol or diethylene glycol. In this initially hypertonic medium the cells at first shrink, but as the solute penetrates and becomes more and more equally distributed between cells and medium, water gradually re-enters and the cells swell toward their original size.

The data required for calculating permeability both to the solute and to water are initial cell volume, V_o, minimal volume attained, V_m, time required to attain this volume, and the ratio $\frac{V_m}{V_o}$.[6] These data in a representative experiment are given in table 2.

TABLE 2.—*Data from a representative experiment for computing permeability values of control and irradiated Arbacia eggs to ethylene glycol* (K_1) *and water* (K_2). *Volumes are in hundreds of cubic micra. The last 2 columns give the values of permeability at a temperature of 24° C. in units stated in table 3, where values for all experiments are summarized*

Cell	Original volume	Minimal volume	Time to minimal volume (min.)	$\frac{V_m}{V_o}$	K_1	K_2
Control	2, 280	1, 863	3. 0	0. 817	3. 3	0. 18
Irradiated	2, 280	1, 875	3. 0	. 822	3. 5	. 18

The results of 8 experiments on determination of permeability to water are given in the designated columns of tables 3 and 4. The mean permeability of the control and of the irradiated cells to water is 0.20 and 0.18, respectively, when ethylene glycol served as the penetrating solute, and 0.21 and 0.22, respectively, when the more rapidly penetrating solute, diethylene glycol, was used. These values are in good agreement. Slightly greater permeability values were obtained by the second method than by the first.[7]

In brief it has been found by two different methods that Roentgen irradiation did not bring about a change in the permeability to water of Arbacia-egg cells.

[6] For details of computation and comments on this method, reference should be made to the original papers of Jacobs (*11*), and of Stewart and Jacobs (*12*). Using this method, they have followed the course of volume changes in Arbacia by direct measurements of individual cells. As they point out, it is difficult by their procedure to determine accurately the initial size of the particular cell whose volume changes they wish to follow. This difficulty is avoided by the use of the diffraction method, which permits measurement of original volume of the particular cells later used in the experiment and, moreover, gives statistical averages of many thousands of individual cells.

[7] This difference is readily explainable on the grounds that by the second method permeability is measured during the course of shrinking, and by the first during the period of swelling of the cells. As has been shown previously for Arbacia eggs, water appears to leave the cell more rapidly than it enters, the calculated values of permeability for exosmosis being somewhat greater than the value for endosmosis (*9*).

TABLE 3.—*Permeability of control and irradiated Arbacia eggs to ethylene glycol and to water. Summary of 4 experiments. The permeability values for ethylene glycol signify the number of mols* $\times 10^{-15}$ *that pass through each square micron of cell surface per minute at a concentration difference of 1 mol per liter, at a temperature of 24° C. For permeability to water the units are given as cubic micra of water per square micron of cell surface per minute per atmosphere of pressure. The values for permeability of control and irradiated cells show no significant differences*

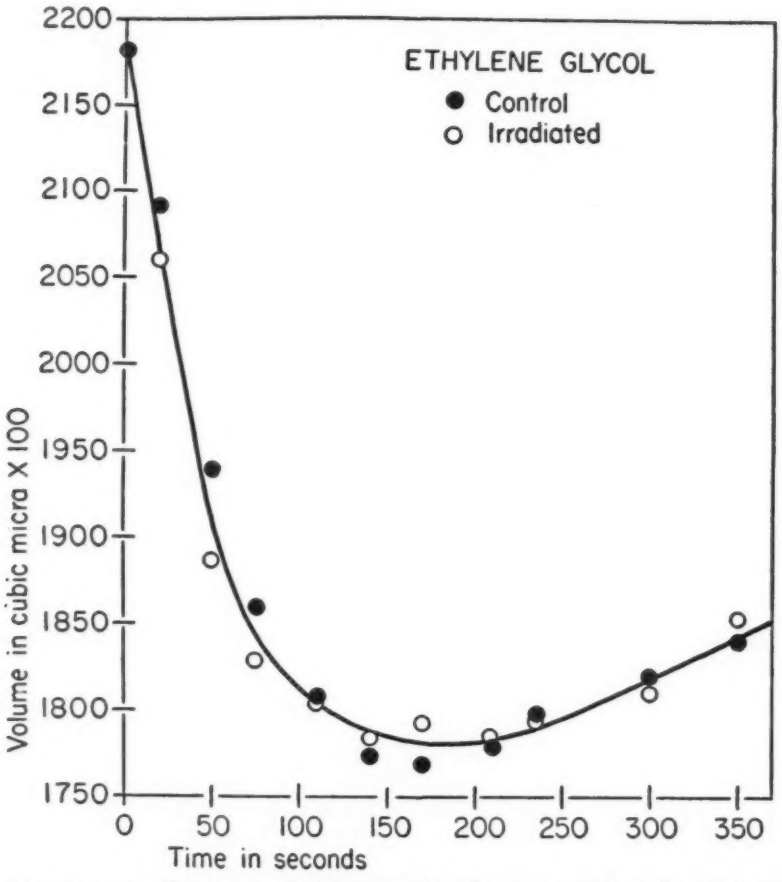
Experiment	Control cells		Irradiated cells	
	Permeability to ethylene glycol $\times 10^{-15}$	Permeability to water	Permeability to ethylene glycol $\times 10^{-15}$	Permeability to water
I	3. 5	0. 20	4. 2	0. 16
II	3. 2	. 21	3. 5	. 17
III	2. 8	. 20	2. 8	. 20
IV	3. 3	. 18	3. 5	. 18
Mean	3. 2	. 20	3. 5	. 18

TABLE 4.—*Permeability of control and irradiated Arbacia eggs to diethylene glycol and to water. Summary of 4 experiments. The units are the same as given in table 3. The values for permeability of control and irradiated cells for both solute and water show no significant differences*

Experiment	Control cells		Irradiated cells	
	Permeability to diethylene glycol $\times 10^{-15}$	Permeability to water	Permeability to diethylene glycol $\times 10^{-15}$	Permeability to water
I	2. 5	0. 22	2. 5	0. 22
II	2. 5	. 22	2. 4	. 24
III	2. 6	. 21	2. 8	. 20
IV	2. 9	. 18	2. 5	. 20
Mean	2. 6	. 21	2. 6	. 22

PERMEABILITY OF THE CELLS TO ETHYLENE GLYCOL

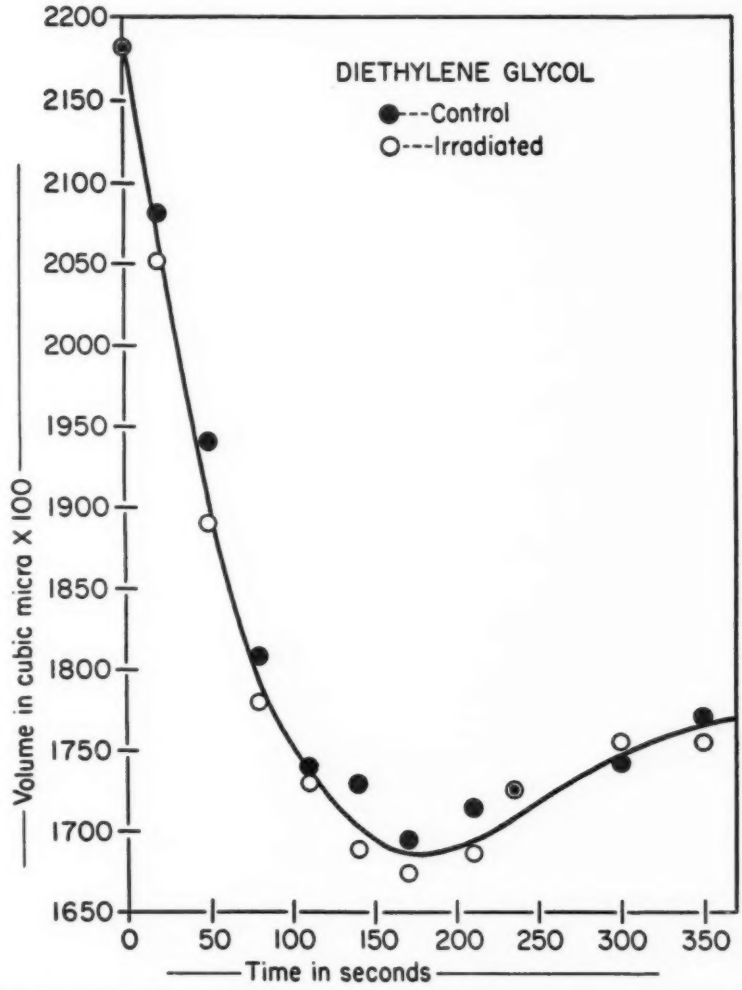
As stated above, the ingenious method devised by Jacobs (*11*) makes it possible to measure the permeability of the same cells to water as well as to a solute. The data required are the same, and are exemplified in table 1. For our first group of experiments we selected ethylene glycol because this substance is known to penetrate Arbacia eggs at a rate convenient for measurement and because it is relatively nontoxic for these cells. The results of 4 experiments are given in table 3. The figures indicate the number of mols $\times 10^{-15}$ of ethylene glycol that pass through each square micron of cell surface per minute, at a concentration difference of 1 mol per liter, at a temperature of 24° C. It is seen that the values for irradiated and control cells are not significantly different. For convenience of inspection, we have averaged the observed volumes of irradiated and control cells from the 4 experiments and plotted them against time (text-fig. 2). For both kinds of cells the observed points are closely scattered about the smooth curve drawn to give the best fit. The figure shows graphically the absence of irradiation effects on permeability to ethylene glycol.



TEXT-FIGURE 2.—The course of shrinkage and subsequent swelling of control and irradiated Arbacia eggs when placed in a solution of 0.5 M ethylene glycol in sea water. The mean volumes from 4 experiments are plotted against time. It is seen that the course of volume change is the same for control and irradiated cells. Permeability values of individual experiments are given in table 3.

PERMEABILITY OF THE CELLS TO DIETHYLENE GLYCOL

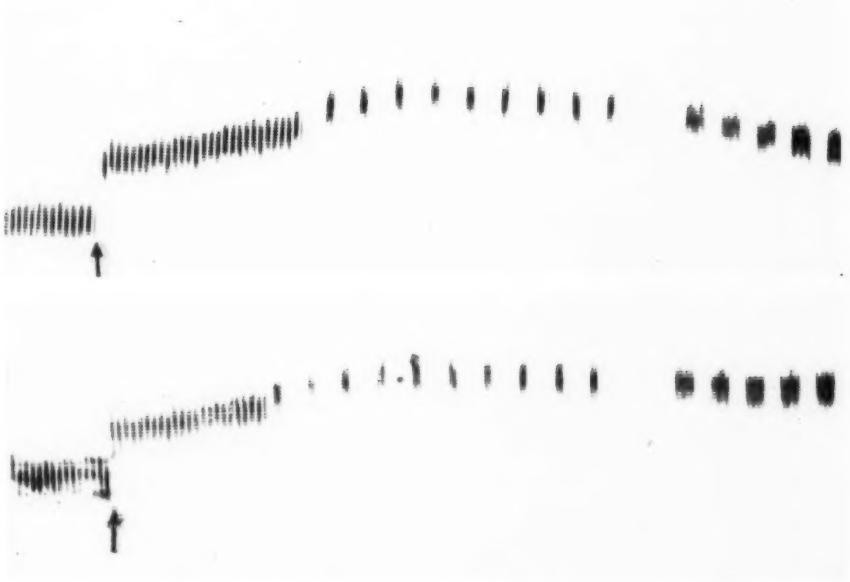
For the second group of 4 experiments we selected the somewhat more rapidly penetrating solute, diethylene glycol. The results are shown in table 4. Again it is seen that exposure to Roentgen rays did not affect the permeability of Arbacia cells. The mean volumes for this solute are the same in the irradiated and the control cells. Similarly text-figure 3, giving the mean volumes of the cells in the 4 experiments plotted against time, graphically shows positive correspondence.



TEXT-FIGURE 3.—The course of shrinkage and subsequent swelling of control and irradiated Arbacia eggs when placed in a solution of 0.5 M diethylene glycol. The mean volumes of 4 experiments are plotted against time. The correspondence of volume changes indicates an absence of irradiation effect. Permeability values of individual experiments are given in table 4.

COMPARISON OF PERMEABILITY OF IRRADIATED AND CONTROL CELLS BY A PHOTOELECTRIC METHOD

In the last group of experiments, a photoelectric method was used. To the cells in sea water was added, at the start of the observations, sufficient pure ethylene glycol to make the environment 1 M with respect to ethylene glycol. This medium is more hypertonic than the one used with the diffraction method, and cell volume changes are more rapid. Repre-



TEXT-FIGURE 4.—Galvanometric records of control (upper graph) and irradiated (lower graph) Arbacia eggs during the course of volume changes in a medium consisting of 1 M ethylene glycol dissolved in sea water. Arrows indicate time of addition of ethylene glycol. The time intervals in the first third of both records are 1 second; in the center, 15 seconds; in the last third, 1 minute. Shrinkage is recorded as increased transmission of light, swelling as decreased transmission. The time required for attaining minimal volume is the same for the irradiated and the control cells, indicating that irradiation has not affected permeability.

sentative galvanometric records are shown for irradiated and control cells in text-figure 4. Here shrinkage is manifest by an increasing amount of light transmitted through the suspension of cells. The time for attainment of minimal volume is the same for both irradiated and control cells. Similar agreement was observed in 3 other experiments. The results obtained by this method are in accord with those obtained by the diffraction method, in that no effect of Roentgen irradiation on permeability was demonstrable.

Comparison of Permeability Values Obtained at Different Times After Irradiation

The permeability values for water and 2 solutes reported have all been derived from measurements made within a short time after the end of irradiation. As already stated, we were particularly interested in more or less immediate, rather than in late, effects of irradiation, and we wished to avoid the complicating effects of recovery. The measurements in the experiments were therefore begun within 10 minutes after the end of exposure, and completed within 30 minutes. In most experiments, however, we repeated measurements after 1 or 2 hours. While we are here not primarily concerned with the values for permeability of these later measurements, it is of interest to record that they did not differ significantly either in degree or in direction from the measurements made earlier.

Effect of Irradiation on Cell Cleavage

A number of investigators have shown that exposure of unfertilized Arbacia eggs causes a delay in the time of occurrence of the first cleavage subsequent to the addition of normal sperm. Henshaw (*7*), whose extensive studies should be consulted for details and for references to the work of others, defines the delay of cleavage as the time required for cleavage in the irradiated eggs over and above the time required for cleavage in the nonirradiated control eggs. In the present experiments, the irradiated eggs were fertilized 3 to 8 minutes after the termination of irradiation. In all experiments cleavage was delayed in over 95 percent of the eggs, the time ranging from 70 to 150 minutes. In each lot there was a small percentage of eggs, usually 1 to 2, less often up to 5 percent, which did cleave considerably ahead of the majority, though not at the same time as the controls. When cleavage did begin, the division rate proceeded in the usual manner, the time schedule of the irradiated eggs, however, always lagging behind the controls. Atypical division forms were common. Eventually many of the irradiated cells reached an abnormal blastula or gastrula stage, and thereafter disintegrated. Within 22 hours all the irradiated eggs had become completely cytolyzed, while the control eggs had developed into normal plutei. The course of events in a representative experiment is shown in a series of photographs (fig. 1).[8]

[8] The photographs were made by Messrs. C. M. Loos and J. C. Robinson in Dr. H. F. Blum's laboratory at Woods Hole, using the apparatus described by him (*8*). By his technique both irradiated and control cells can be photographed at the same time, and without disturbing the positions of the individual cells.

DISCUSSION

It must at once be emphasized that the results obtained with Arbacia eggs cannot, without experimental evidence, be applied to all cells. Bearing this in mind, our conclusions may be stated as follows:

The experiments have shown that exposure of unfertilized Arbacia eggs to 100,000 roentgen units under the conditions stated does not affect the permeability of these cells either to water or to the penetrating solutes, ethylene glycol and diethylene glycol. However, these irradiated cells, when inseminated, reveal great alterations: the time to first cleavage is delayed, many cells divide atypically, and within 24 hours practically all disintegrate. Evidently the effect of irradiation upon the cells is differential: the mechanism governing cell permeability remains unchanged. If we assume that the cell surface is chiefly concerned with permeability, and the nucleus with cleavage mechanism, we should have to infer that irradiation promptly leads to damage of nuclear components, but not simultaneously to components of the plasma membrane. Such inference, however, is perhaps not as yet fully warranted. The action of radiation on living cells is exceedingly complex and it is by no means certain which cell components are primarily affected, or how they are affected.[9] We must, therefore, content ourselves at present with recording our experimental results, and point out that factors other than Roentgen irradiation can exert similar differential effects on Arbacia eggs.

In previous experiments it was found that permeability to water and to various solutes of Arbacia cells (as well as of the egg cells of Chaetopterus, and the erythrocytes of mouse and beef) was not altered after exposure to choleic acid compounds of several carcinogenic hydrocarbons and related noncarcinogens. Under the same conditions the carcinogens did, however, induce decided disturbances of cell cleavage, whereas the related noncarcinogens did not affect the cells adversely (*18*). Similarly, experiments with heavy water (99.5 percent D_2O) indicated that this substance did not change the properties of the plasma membrane of Arbacia eggs so far as permeability is concerned; the values of permeability to D_2O were the same as those for H_2O. But 99.5 percent D_2O "sea water" completely inhibited cell cleavage when eggs inseminated in ordinary sea water were transferred to this medium. The cells failed to develop sperm asters, indicating that development was inhibited within several minutes after the fertilized eggs came in contact with heavy water (*19*). Thus, the differential effects of irradiation on cell cleavage and on permeability of Arbacia eggs are not unique, but can be produced by other factors.

These results have a bearing upon an important concept of biology, namely injury. Like some other fundamental biological concepts, injury is difficult to define with precision (*20*). An exact definition of injury

[9] For a discussion of the mode of action of irradiation, reference should be made to recent publications by Lea (*13*), Zirkle (*14*), Giese (*15*), Spear (*16*), and Duryee (*17*).

can be made only in terms that are quantitative and that refer to definable biological or physical criteria. Suitable criteria, as for example in the present experiments, are cell cleavage and cell permeability. It is generally accepted that interference with cleavage signifies injury. That this process is irreversible is indicated in the present experiments by the actual disintegration of the cells once they have passed through a few division stages. But injury is said to induce an increase in cell permeability. There is no doubt that this is true of many kinds of injury. For example, we ourselves have found that injury as revealed by alteration of cell cleavage, and as induced by high temperature, anisotonic solutions and certain narcotics, causes marked increase in the permeability of Arbacia eggs to water. Furthermore, this increase can be correlated with the degree of injury and hence may be taken as a measure of injury (*21*). The present experiments make it clear, however, that there are forms of injury (as defined by abnormal cleavage) in which permeability to water and to the solutes studied is not altered. Irradiation, certain carcinogens, and D_2O are among factors that exert such differential effects. The significance of these conclusions on cells in general must await further exploration.

SUMMARY

To determine the effect of Roentgen rays on permeability, the unfertilized egg of the sea urchin, *Arbacia punctulata*, was selected. Utilization of this cell permits accurate measurements of permeability and readily recognizable indications of injury. Permeability to water, to ethylene glycol, and to diethylene glycol was measured within 10 to 30 minutes after irradiation. It was found in each instance that the irradiated cells did not differ in their permeability values from the control cells. However, that the irradiation proved injurious to the cells was shown, for, after fertilization, cleavage was delayed and atypical, and later the cells cytolyzed.

The effect of irradiation is therefore differential with respect to two functions of the cells: cleavage and permeability. Such differential effect is not unique for irradiation but is similar to the action of certain other factors such as some carcinogens and D_2O. These results are discussed with respect to their bearing on the general biological concept of injury.

REFERENCES

(*1*) Heilbrunn, L. V., and Mazia, D.: The action of radiations on living protoplasms. *In* Duggar, B. M. (Ed), Biological Effects of Radiation. McGraw-Hill, New York, 1936.

(*2*) Ting, T. P., and Zirkle, R. E.: Effects of x-rays on the permeability of erythrocytes to water and certain non-electrolytes. J. Cell. & Comp. Physiol. 16: 269–276, 1940.

(*3*) Reed, E. A.: Ultraviolet light and permeability of sea urchin eggs. J. Cell. & Comp. Physiol. 31: 261–288, 1948.

(4) LUCKÉ, B., HARTLINE, H. K., and RICCA, R. A.: Comparative permeability to water and to certain solutes of the egg cells of three marine invertebrates, Arbacia, Cumingia and Chaetopterus. J. Cell. & Comp. Physiol. 14: 237–252, 1939.

(5) LUCKÉ, B., LARRABEE, M. G., and HARTLINE, H. K.: Studies on osmotic equilibrium and on the kinetics of osmosis in living cells by a diffraction method. J. Gen. Physiol. 19: 1–17, 1935.

(6) PARPART, A. K.: The permeability of the mammalian erythrocyte to deuterium oxide (heavy water). J. Cell. & Comp. Physiol. 7: 153–162, 1935.

(7) HENSHAW, P. S.: Further studies on the action of Roentgen-rays on the gametes of Arbacia punctulata (I–V). Am. J. Roentgenol. 43: 899–933, 1940.

(8) BLUM, H. F., and PRICE, J. P.: Delay of cleavage of the Arbacia egg by ultraviolet radiation. J. Gen. Physiol. 33: 285–304, 1950.

(9) LUCKÉ, B., HARTLINE, H. K., and MCCUTCHEON, M.: Further studies on the kinetics of osmosis in living cells. J. Gen. Physiol. 14: 405–419, 1931.

(10) LUCKÉ, B., and RICCA, R. A.: Osmotic properties of the egg cells of the oyster (Ostrea virginica). J. Gen. Physiol. 25: 215–227, 1941.

(11) JACOBS, M. H.: The simultaneous measurement of cell permeability to water and to dissolved substances. J. Cell. & Comp. Physiol. 2: 427–444, 1933.

(12) STEWART, D. R., and JACOBS, M. H.: Further studies on the permeability of the egg of Arbacia punctulata to certain solutes and to water. J. Cell. & Comp. Physiol. 7: 333–350, 1936.

(13) LEA, D. E.: Action of radiations on living cells. Cambridge Univ. Press, 1947.

(14) ZIRKLE, R. E.: Relationships between chemical and biological effects of ionizing radiations. Radiology 52: 846–855, 1949.

(15) GIESE, A. C.: Radiations and cell division. Quart. Rev. Biol. 22: 253–282, 1947.

(16) SPEAR, F. G. (Ed.): Certain aspects of the action of radiation on living cells. Brit. J. Radiol., suppl. 1, Brit. Inst. Radiol. London, 1947.

(17) DURYEE, W. R.: The nature of radiation injury to amphibian cell nuclei. J. Nat. Cancer Inst. 10: 735–796, 1949.

(18) LUCKÉ, B., PARPART, A. K., and RICCA, R. A.: Failure of choleic acid compounds of carcinogenic hydrocarbons to alter permeability of marine eggs and of mammalian erythrocytes. Cancer Research 1: 709–713, 1941.

(19) LUCKÉ, B., and HARVEY, E. N.: The permeability of living cells to heavy water (deuterium oxide). J. Cell. & Comp. Physiol. 5: 473–482, 1935.

(20) OSTERHOUT, W. J. V.: Injury, Recovery and Death in Relation to Conductivity and Permeability. J. B. Lippincott, Philadelphia, 1922.

(21) LUCKÉ, B., and MCCUTCHEON, M.: The effect of injury on cellular permeability to water. Arch. Path. 10: 662–670, 1930.

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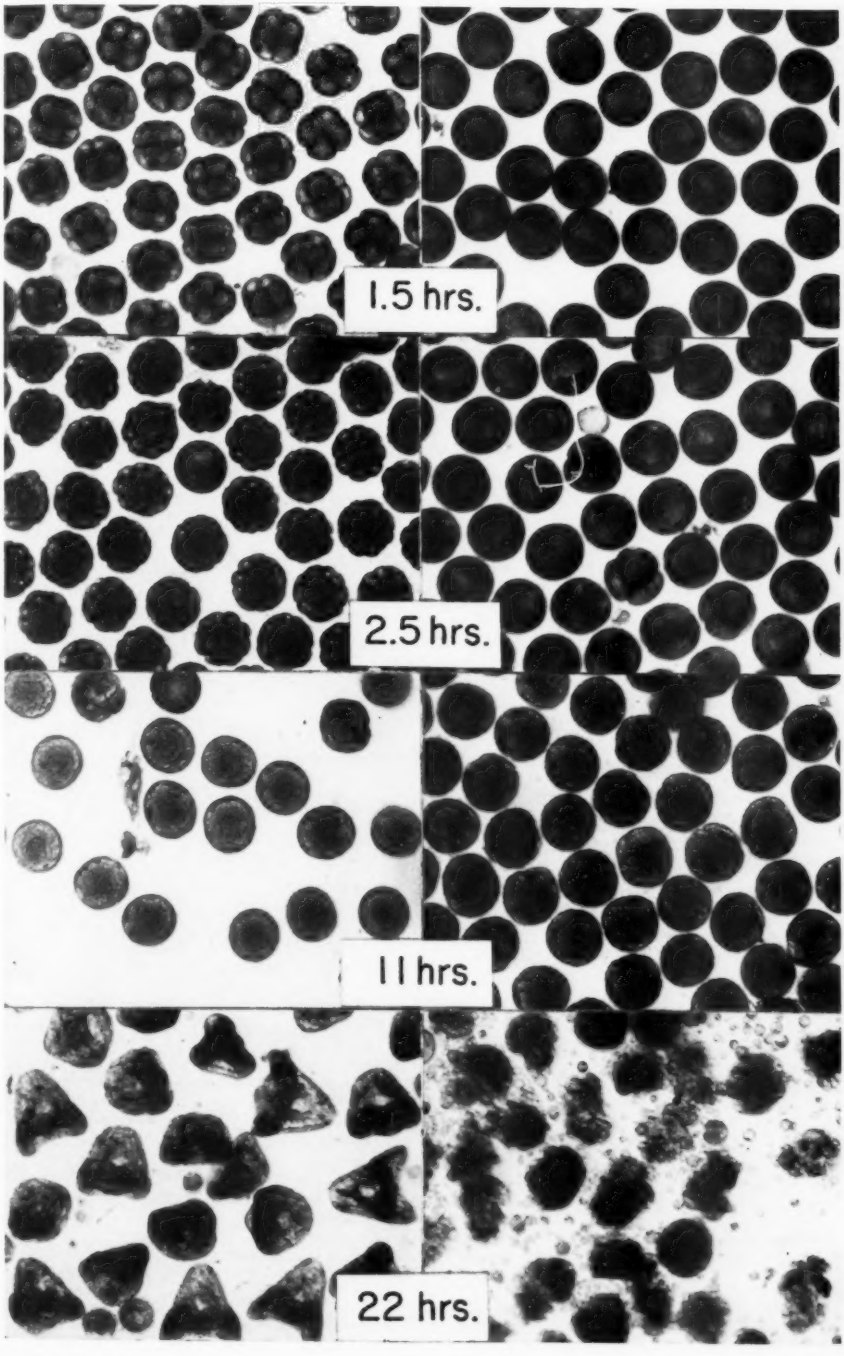
FIGURE 1.—A selected group from an extensive series of photographs of control and irradiated Arbacia eggs, showing effects of irradiation on cell cleavage. The figures indicate time elapsed in hours following introduction of normal sperm. On the left side are the control eggs, on the right the irradiated eggs. It is seen that 1.5 hours after insemination the control eggs have reached the 4 or 8 cell stage, whereas in nearly all the irradiated eggs cleavage has not as yet begun. After 2.5 hours, the controls have reached a multicellular stage, while most of the irradiated eggs are still undivided; atypical division is shown among those that have started cleavage. After 11 hours, all of the controls are in the free-swimming stage, and many have left the field, whereas division of the irradiated eggs has progressed to blastulae that are slowly revolving. In 22 hours the controls have developed into plutei, while the irradiated eggs have disintegrated.


1.5 hrs.
2.5 hrs.
11 hrs.
22 hrs.

THE SPONTANEOUS BIOPSY[1,2]

GEORGE ADAMS, M. D., M. P. H. *Sr. Surgeon, U. S. Public Health Service, Chief, Laboratory Section, Cancer Investigation Center, Hot Springs National Park, Arkansas. Associate Professor of Preventive Medicine and Public Health, University of Arkansas School of Medicine, Little Rock, Ark.*

It has been the general impression that the cytologic method of cancer detection is something new. The extent of the newness—Papanicolaou's first paper (*4*) dealing with the cytology of cancer was entitled "New Cancer Diagnosis"[3]—and the importance of the contributions of Papanicolaou and Traut, Dudgeon, Mosto, Graham, Ayre, Herbut, and others become apparent when placed in their historic setting. It need hardly be emphasized that the early history of the subject has been discontinuous. There were sporadic reports by isolated investigators that awakened an alertness in others. Variants of fixing, embedding, and staining methods were reported by different men in different countries, often without knowledge of similar work in progress or completed elsewhere.[4] This is, of course, a familiar phenomenon in the history of science. The interest was

[1] Received for publication January 16, 1951.

[2] This expression occurs in a paper by Lamarque *et al.* (*1*) entitled "Biopsie spontanée d'un cancer secondaire du poumon." They apply the term to the ejection of nonsurgical "tumor fragments large enough to permit of exact histologic examination." It is used also in a paper by Brachetto-Brian (*13*). I am limiting the discussion to documented case reports, supported by study of histologic sections.

[3] Papanicolaou more recently (*5*) uses the word "revival," "because the idea of examining various body fluids * * * for the purpose of detecting cancer cells is not new." In spite of the modern aversion to the use of eponyms, the terms "Papanicolaou method" and "Papanicolaou stain" are probably here to stay. Their use appears justified as a recognition of the important labors of the protagonist.

Vaginal cytology has been examined for more than a century. As early as 1847 Pouchet (*6*) reported his independent microscopic studies of the menstrual cycle as reflected in the vaginal secretion obtained at the vulva. Drawings in his Atlas (*7*) of unstained preparations of cyclical stages reveal marked observational skill. Dickinson (*21*) as early as 1869 studied vaginal secretion for evidence of diagnostic cellular criteria in cases of known uterine malignancy. His failure to find "anything diagnostic of the disease" illustrates an inherent, admitted weakness of the cytologic method in cases of far-advanced, necrotic malignant lesions. However, it was not until about 80 years later that Papanicolaou first recognized exfoliated cancer cells in vaginal secretion and adapted the method vigorously to its most productive field.

[4] Luecke and Klebs (*8*) as early as 1867 described malignant cells in smears of peritoneal fluid obtained by trocar in a case of carcinoma of the pancreas with metastases to the omentum. Ferguson (*22*) in 1893 described the alcohol fixation, celloidin embedding, sectioning, and staining of urinary sediment for the detection of tumor cells. Three years later, Bahrenburg (*9*) described the "fixing and hardening and studying of cellular elements of cavity fluids," and was able to report "epithelial elements which were * * * highly suggestive, if not conclusive evidence of carcinoma involving the peritoneum" in paraffin embedded semicoagula of ascitic fluid. In 1932 Morin (*10*) rediscovered the fixing, sectioning, and study of cavity fluids as a diagnostic tool. The alleged priority of his method was caustically attacked by Mosto (*16*). The important early work on the cytology of sputum and bronchial secretions in English-speaking countries appears to have been done without firsthand acquaintance with the experience of a group of South American investigators (*13–18*), who also used classical methods of embedding and sectioning.

there; the findings were striking; the impetus of a devoted, painstaking scientist was lacking to elevate the subject to consistent scientific duplicability.

The earliest report of a spontaneous biopsy with microscopic confirmation of the ejected tumor mass is probably that of L. S. Beale (*19*)[5] who in 1860 reported the case of a 44-year-old woman with "cancer of the pharynx and adjacent parts." The patient's sputum when examined microscopically "without the addition of water or any chemical reagent," showed "various forms of the so-called cells in the sputum" which were "clearly not cells, but fragments of a mass containing, irregularly scattered through it, nuclei or small cells." While Beale's unconventional interpretation enabled him to make a diagnosis verified at autopsy, the bizarre forms pictured in his woodcut suggest rather those of epidermoid carcinoma.

The next reference to a case of spontaneous biopsy is one to gross particles observed in the urine of a 43-year-old male reported by Sanders (*20*) in 1864. The patient's "cancer was of great extent, involving the whole interior of the bladder, and obstructing completely the left ureter. The most important points in the case were, that there was no hematuria at any time, and that the patient passed *per urethram* fragments of the cancer sometimes of small size like mere shreds, at other times forming cylindrical plugs about an inch long, which often became impacted in the urethra, and obstructed the flow of urine. Examined under the microscope, these plugs consisted of epithelial cells more or less disintegrated, among which could be observed peculiar concentric globular bodies." He describes these bodies as having a "central part enveloped by flattened epithelial scales forming peripheral concentric layers * * * The post-mortem examination showed clearly that these concentric corpuscles belonged to the cancerous structure, where they could be found in large numbers."

Of particular historic interest because it is apparently the first report of the spontaneous biopsy of a relatively "early," clinically unsuspected, cancer is Dickinson's (*21*) case report of 1869. His 51-year-old, previously asymptomatic, male patient had passed "a small quantity of bloodtinged urine, which contained some small, buff-colored lumps, which had very much the appearance of fibrinous coagula, for which they were at first mistaken. These masses had drawn attention by briefly obstructing the urethra during their passage * * * The small quantity of urine in which the masses lay abounded with large cells, presenting a great variety of shapes; some were spheroidal, some spindle-shaped, others irregularly

[5] As early as 1846 Walshe in *The Nature and Treatment of Cancer* (p. 350) cites references for the expectoration of "encephaloid detritus" in cases of alleged malignant lesions of the lungs. It is obvious, however, that his sources refer to gross rather than microscopically verified diagnoses. Thus Greene (*2*) in 1843 discussed a case with "caseous matter in the expectoration," while autopsy revealed "a remarkable specimen of malignant disease within the cranium and thorax." Lancereaux's (*3*) case reported in 1858 and frequently quoted in the French literature as an example of the diagnosis of pulmonary cancer made *intra vitam*, fulfills the conditions of a spontaneous biopsy, which, however, apparently was not subjected to histologic proof. His patient was a 49-year-old laundress who expectorated matter "about the size of a nut" and "in all respects analogous to cerebral substance." Autopsy revealed extensive primary pulmonary neoplasm and metastases of an "encephaloid" nature.

angular, and curved * * * There could be no doubt that the masses described formed parts of an organized cellular growth, such as would belong to a malignant tumor, apparently of the encephaloid kind." The author reasoned that "under the circumstances there was no difficulty in inferring, from the evidence of what had passed by the urethra, that the patient had a malignant tumor, probably of the encephaloid variety, that it was situated in the bladder, and that it was of a kind which might be expected to grow rapidly. But so complete was the absence of bladder symptoms, and so perfect the apparent health of the patient, that this view of the case was received with the greatest surprise, and at first almost with incredulity." He, nevertheless, "ventured * * * to predict that the patient had seen his last summer." Death did occur 8 months after the first examination. Commenting on the extreme rarity of such a diagnosis Dickinson counseled that "in cases, indeed, in which malignant disease of the bladder or kidney is suspected, the microscope is often a source of error. Under circumstances of irritation and disturbance large cells, presenting quite the idea of cancer cells, of all shapes and characters, regular and irregular, rounded, angular, and elongated, are occasionally thrown off in abundance from various parts of the urinary membrane. Patients who upon the evidence of urine presenting such appearances have been convicted of cancer have been known to get well and remain so." Following the prevalent Müller-Virchow insistence on the nonspecificity of cytologic criteria of malignancy, he remarked that "no isolated or detached cells, therefore, found in the urine, can be regarded as proof of cancer in the urinary tract, while the absence of such cells presents no argument in favor of the freedom of the bladder and kidney from malignant growth." [6]

Hampeln (*23*) appears to have been the first to report a sarcomatous lung metastasis primarily diagnosed by sections of an expectorated mass. In 1876 he described the case of a 19-year-old male who 3 years earlier had had an amputation because of osteosarcoma of the left lower femur. The patient had definite clinical findings referable to his lungs, and expectorated grayish-red firm tumor particles up to one half inch in length. A diagnosis of "round cell sarcoma" was made on the basis of microscopic studies of embedded tumor fragments.

Huber (*24*) in 1889 presented the case of a 21-year-old Bulgarian law student whose painful left-leg lesion was attributed to arthritis or rheumatism before its malignant nature was suspected. The author reports that months before the patient's death expectorated masses were diagnosed as portions of sarcomatous metastases "with the greatest imaginable certainty." Autopsy revealed a massive local lesion with metastases to the lung.

Troisier (*25*, *26*) in the same year described the case of a 34-year-old male with cancer of the suprarenal cortex metastatic to the lungs.

[6] Ferguson (22), however, as early as 1893 "believed that the best method of making a diagnosis of tumor of the bladder was by continuous microscopical examination of the urine."

After a violent access of coughing the patient expectorated a small, fleshy, whitish mass the size of an orange seed. The histologic findings of the expectorated mass were in essential accord with those of the autopsy.[7]

While the first report of primary carcinoma of the lung diagnosed by microscopic study solely of the cytologic components of sputum appears to be that of Ménétrier (*27*)[8] in 1886, the first report of a primary bronchial carcinoma diagnosed *intra vitam* from sections of grossly visible expectorated tumor masses is probably that of Ehrich's (*28*) doctoral dissertation of 1891. His patient was a 51-year-old male who after several years of bronchial catarrh and increasing hemoptyses developed intense right-sided thoracic pain and during a severe paroxysm of coughing expectorated irregular tissue masses. These on microscopic examination revealed numerous clumped pleomorphic cells, with a tendency to concentric arrangement, although no actual cornification was demonstrable. According to the author a diagnosis of pulmonary carcinoma could be made "with absolute certainty" on the basis of microscopic sections of the expectorated masses. The diagnosis was confirmed at autopsy, which revealed "carcinoma pulmonis dextrae et pleurae" originating in the right main bronchus.

Feldt (*31*) in 1903 contributed two additional cases to the subject of spontaneous biopsies occurring in the sputum. The first was that of a 46-year-old Frenchwoman who repeatedly expectorated whitish tumor fragments up to 20 mm. in length and 3 mm. in thickness, which on microscopic examination were found to be compatible with a diagnosis of "round cell sarcoma." His second case was that of a 16-year-old male who had had an amputation because of osteosarcoma of the tibia. During the last weeks of this patient's life there occurred profuse hemoptyses. Two ejected cylindrical masses measuring 1.5 cm. in length and 0.5 cm. in thickness on microscopic examination revealed the histologic picture of a very vascular "giant cell sarcoma."

In 1907 Chace (*32*) reported on the "Diagnosis of Carcinoma of the Stomach from a Fragment of the Tumor Obtained by Means of the Stomach Tube." He considered his observation "peculiarly interesting because a large piece of tissue was drawn through the tube during quiet lavage in a case where the tumor was freely movable and no larger than a walnut."

[7] A very similar case was reported by Nussbaum (*37*) in 1922 (*vide infra*).

[8] Credit for the first report of primary carcinoma of the lung diagnosed by microscopic study of expectorated tumor cells has been assigned to Hampeln (*29*), who published his case report in 1887. At autopsy of his 62-year-old patient a pulmonary lesion was demonstrated which was classified histologically as "carcinoma alveolare." "Of even greater significance," states Hampeln, "were the microscopic findings of the sputum." An examination made about 5 months before death yielded "besides detritus and small, delicately granular roundcells, numerous pleomorphic granular clubshaped and spindleshaped cells of remarkable size with single or several nuclei." To the author, the malignant nature of these cells was "beyond doubt." Priority, however, should probably be accorded to Ménétrier whose report antedates that of Hampeln by a year. Repeated microscopic search of sputa of his 51-year-old male patient eventually revealed small whitish lumps which showed "irregular epithelial elements with very large nucleus, resembling those of a carcinoma." Autopsy disclosed a malignancy compatible histologically with a diagnosis of epidermoid carcinoma. Betschart's (*30*) classical report of a case with primary pulmonary carcinoma diagnosed *intra vitam* by cytologic criteria did not appear until 1895.

The tumor fragment recovered from his 43-year-old male patient was a "moderately hard, flesh-colored, irregularly-shaped mass, about 1 by 1.5 by .25 centimeters." He interpreted the histologic structure as that of an "adenocarcinoma presenting marked colloid degeneration," and concluded that "if a more minute examination of the wash-water were made in cases of gastric lavage, we would possibly be able to make not only an earlier but an exact diagnosis of gastric conditions."

Pfahler (*36*) in 1921 in a discussion of Bryan's paper on the "Roentgenological Study of Primary 'Lung Carcinomata,'" relates the case of a physician-friend who "coughed up a part of the tumor and had sense enough to take it to a pathologist for examination." Treatment resulted in "an almost complete disappearance of the tumor but later the patient died of some pulmonary condition," presumably of carcinoma.

Nussbaum (*37*) in 1922 reported a case very similar to Troisier's (*vide supra*). He was able to make a diagnosis of malignancy *intra vitam* in a 68-year-old foreman on the basis of stained sections of embedded expectorated bean-to-filbert-sized tumor fragments. Microscopically these were characterized by central regressive fatty and necrotic changes. Autopsy revealed carcinoma of the left suprarenal gland with pulmonary metastasis.

In the same year Barker (*38*) published the case of a 54-year-old white male who had coughed up a "mass" a few weeks earlier said to have been "about the size of the end of his thumb." A similar mass expectorated during the patient's stay in the hospital was reported by the pathologist as "neoplastic, consisting chiefly of stratified epithelium, characteristic of one form of primary carcinoma of the bronchus." Death occurred 4 weeks after this spontaneous biopsy.

In 1931 Sergent *et al.* (*11*) described the case of a 49-year-old male, who expectorated a small compact grayish mass the size of an acorn. At first the symptomatology suggested a cold abscess of bone with pleural effusion. A diagnosis of "round cell sarcoma" was made on the basis of aspiration of pleural fluid and biopsy of an involved gland. Shortly before death another tumor fragment was ejected through coughing. It is not clear whether the expectorated fragments also were subjected to histologic examination.

Jekel (*39*) in 1936 reported the case of a 56-year-old white male patient who "presented a confusing clinical picture of possible carcinoma of the stomach without a palpable mass." In the absence of conclusive radiographic studies a definite diagnosis of adenocarcinoma was made after incidental aspiration of a particle of tissue through a stomach tube during routine gastric analysis. In spite of the inoperability of the case Jekel suggests that "nevertheless, the possibility of an *early* diagnosis in this manner must be admitted." [9]

[9] Chace's (*32*) and Jekel's (*39*) cases are not strictly "spontaneous" such as might, perhaps, be afforded by vomiting or hemoptysis, but are rather "fortuitous" biopsies depending on the trauma of friction and suction. The purely cytologic aspects of gastric cancer have been explored more extensively by Mosto (*17*), Richardson (*33*), Gladstone (*34*) and Panico *et al.* (*35*). The last two particularly use ingenious methods of inducing exfoliation, Gladstone by attaching portions of gelfoam sponge to the stomach tube, Panico *et al.*, by the use of an inflated balloon with roughened surface.

In 1936 Lamarque *et al.* (*1*) reported the "spontaneous biopsy" of a pulmonary metastasis of a malignant axillary tumor. Their case was that of a 20-year-old miner, who had an axillary mass extending to the pleura and lung. During a violent attack of coughing the patient ejected a cylindrical fragment of tissue two or three centimeters in length resembling a large piece of crayon. Because of the excellent preservation—"a biopsy made with forceps could not have produced better preserved fragments"—an exact histopathologic diagnosis was possible. This revealed "atypical proliferation of mesenchymatous elements with considerable amitotic and mitotic activity * * *" There were numerous blood spaces of the sarcomatous type, that is, their lumina lacked true endothelial lining and were marginated directly by the sarcomatous cells, which appeared to be forming fibrils.

Brachetto-Brian (*13*) in 1937 reported a case seen by him in 1919. The patient was a 55-year-old male who complained of difficulty in breathing, inability to sleep on his back or his left side, and a cough accompanied by hemoptysis of two months' duration. A few days later during a severe attack of asphyxia he expectorated tumor fragments the size of filberts. Histologic examination of these spontaneous biopsies revealed them to be epitheliomatous. A year and a half after the first examination the left lung was extensively infiltrated.

While evidence of pulmonary metastases from clear-cell carcinoma of the kidney has been reported from sputum analysis (*40*), there appears to be no reference in the literature to the expectoration of grossly visible, biopsy-like fragments of such metastases. Therefore, it may be of interest to recall here a case of my own (*41*) where an expectorated tumor fragment met Lamarque's criteria of a spontaneous biopsy. It affords an interesting counterpart to those of Troisier and Nussbaum. The material sent to my laboratory was labeled sputum, but contained a cylindroid piece of tissue measuring 3.3 by 1.4 by 1.3 cm. It was moderately firm, irregular, and roughened, and consisted of grayish-white strands interposed between areas of dark brown, slightly softer material. Along one margin there was soft, brown, friable material, apparently clotted blood. Histologic sections revealed cellular morphology and architecture consistent with that of clear-cell carcinoma of the kidney (fig. 1).[10] It was then ascertained that the patient had undergone nephrectomy several years earlier, which had demonstrated the presence of clear-cell renal carcinoma, with plugging of the renal vessels by tumor tissue. Sections of the expectorated fragment were indistinguishable from those of the original tumor (fig. 2).[11]

SUMMARY

A search of the literature for documented case reports of so-called spontaneous biopsies has yielded 17 examples conforming to Lamarque's

[10] S–14953, Laboratory, U. S. Marine Hospital, New Orleans, La.

[11] S–8362, Laboratory, U. S. Marine Hospital, New Orleans, La., original diagnosis by Dr. Chapman Binford.

definition, *i. e.*, the ejection of nonsurgical "tumor fragments large enough to permit of exact histologic examination." They comprise a variety of anatomical sources—pharynx, lungs, bladder, stomach. Included among them are primary and metastatic carcinomas as well as metastatic sarcomas. Although modern cytology has achieved its most signal successes in the study of vaginal secretion, there is apparently a complete absence of pertinent case reports relating to the female genital tract. There is on the other hand a preponderance of primary and secondary tumors of the lung. It is, of course, impossible to determine the actual frequency with which such diagnoses have been made in general practice: Brachetto-Brian (*13*) reported his case 18 years after the diagnosis was made; Jekel (*39*) refers to an apparently unpublished case of Stout's. There were a few earlier reports without histologic confirmation, and there is an occasional undocumented reference in monographs and textbooks. The relatively few satisfactory case reports, however, and the emphasis that successive authors place on the apparent rarity of such a diagnosis, would make such cases medical curiosities rather than examples of a statistically important means of diagnosis. With the exception of Dickinson's case the patients' lesions were far advanced. The attendant necrosis and erosion that facilitated ejection, at the same time made histologic diagnosis difficult. Co-existing ancillary signs and symptoms no doubt influenced the diagnosis then even as now with our more refined techniques. Nevertheless, the elements of a method are there: the diagnoses were made *intra vitam;* they were made on tumor fragments spontaneously ejected through natural passages. The malignant nature, if not their correct specific type, was established by microscopic examination. As precursors of our present methods of exfoliative cytology their educative value is evident. There are repeated admonitions in the reports to be alert for such evidences of malignancy, and the possibility of their use in the early detection of cancer is emphasized. If such case reports accomplished nothing else, they prepared the way for the extensive search of the last two decades for a clinically dependable cytodiagnostic tool.

REFERENCES

(*1*) LAMARQUE, P., GUIBERT, L., BETOULIERES, P., and BOUGAREL: Biopsie spontanée d'un cancer secondaire du poumon. Arch. Soc. d. sc. méd. et biol. de Montpellier 17: 351–359, 1936.

(*2*) GREENE: Encephaloid tumors in the brain and lungs. Dublin J. Med. Sc. 24: 282–283, 1843.

(*3*) LANCEREAUX, M.: Cancer encephaloïde primitif du poumon, généralisé dans le foie, la rate et les reins. Bull. Soc. anat. 3: 515–520, 1858.

(*4*) PAPANICOLAOU, G. N.: New cancer diagnosis. Proc. Third Race Betterment Conference, Battle Creek, Mich. 528–534, 1928.

(*5*) ———: Cytologic diagnosis of uterine cancer by examination of vaginal and uterine secretions. Am. J. Clin. Path. 19: 301, 1949.

(*6*) POUCHET, F.-A.: Théorie positive de l'ovulation spontanée et de la fécondation des mammifères et de l'espèce humaine. Paris, Esp. pp. 240–256, 1847.

(*7*) ———: ATLAS to accompany (*6*). Paris. Esp. plates XII and XIII, 1847.

(8) LUECKE, A., and KLEBS, E.: Beitrag zur Ovariotomie und zur Kenntnis der Abdominalgeschwülste. Virchow's Arch. 41: 10–12, 1867.

(9) BAHRENBURG, L. P. H.: On the diagnostic results of the microscopical examination of the ascitic fluid in two cases of carcinoma involving the peritoneum. Cleveland Med. Gazette 11: 274–278, 1896.

(10) MORIN, J. E.: La technique anatomo-pathologique dans le diagnostic étiologique des liquides d'épanchement. Arch. méd. chir. de l'app. respir. 7: 221–232, 1932.

(11) SERGENT, E., MIGNOT, R., DURAND, H., KOURILSKY, R., and BENDA, R.: Formes cliniques et diagnostic du cancer primitif du poumon. Arch. méd. chir. de l'app. respir. 6: 527–566, esp. Obs. VIII, 536–537, 1931.

(12) VITON, J. J., VIVOLI, D., and MARANO, A.: La inclusión de esputos en el diagnóstico de las enfermedades pulmonares. Semana méd. 2: 234–237, 1934.

(13) BRACHETTO-BRIAN, D.: El valor de la inclusión de esputos en el diagnóstico del cáncer del pulmón. Rev. Asoc. méd. argent. 51: 237–240, 1937.

(14) BENCE, A. E.: El lavado bronquial en el diagnóstico precóz del cáncer del pulmón. Semana méd. 2: 836–839, 1940.

(15) GAMBA, R., and LAMBERTI, C. E.: La inclusión de esputos en el diagnóstico de cáncer del pulmón. Presna méd. argent. 23: 2711–2712, 1936.

(16) MOSTO, D., and POLAK, M.: Valor de la inclusión de esputos para el diagnóstico del cáncer del pulmón. Rev. Asoc. méd. argent. 51: 97–103, 1937. Essentially the same article appeared also in Semana méd. 1: 1549–1554, 1937.

(17) ———: Valor del exámen histopatológico del jugo gástrico incluído en parafina, en el diagnóstico del cáncer de estómago. Día méd. 10: 295, 1938.

(18) ———: Valor de la inclusión de líquidos de secreción, exudación y transudación en el diagnóstico del cáncer. Rev. méd. y cien. afines. 2: 490–504, 1940.

(19) BEALE, L. S.: Examination of sputum from a case of cancer of the pharynx and adjacent parts. Arch. Med. 2: 44–46, 1860.

(20) SANDERS: Cancer of the bladder. Fragments forming urethal plugs discharged in the urine. Concentric colloid bodies. Edinburgh Med. J. 111: 273–274, 1864.

(21) DICKINSON, W. H.: Portions of a cancerous growth passed by the urethra. Trans. Path. Soc. 20: 233–237, 1869.

(22) FERGUSON, F.: The diagnosis of tumors of the bladder by microscopical examination. Proc. N. Y. Path. Soc. 71–73, 1893.

(23) HAMPELIN, P.: Sarcom der Lunge. St. Petersb. med. Wschr. 40: 1–2, 1876.

(24) HUBER, A.: Ueber Lungensarkom. Ztschr. klin. Med. 17: 341–353, 1889.

(25) TROISIER, J.: Ganglion sus-claviculaire gauche dans un cas de cancer de la capsule surrénale avec propagation au canal thoracique, et lymphangite pulmonaire généralisée. Bull. et Mém. Soc. Méd. Hôp. 7: (3d series) 266–268, 1890.

(26) ———: Cancer du canal thoracique. Bull. et Mém. Soc. Méd. Hôp. 14: (3d series) 302–317, 1897.

(27) MÉNÉTRIER, P.: Cancer primitif du poumon. Bull. Soc. Anat. 61: (4th series, vol. 11) 643–647, 1886.

(28) EHRICH, W.: Ueber das primäre Bronchial und Lungencarcinom. Diss. Marburg, 1891.

(29) HAMPELN, P.: Ueber einen Fall von primärem Lungen—Pleura—Carcinom. St. Petersb. med. Wschr. 12: (new series, 4) 137–139, 1887.

(30) BETSCHART, E.: Ueber die Diagnose maligner Lungentumoren aus dem Sputum. Virchow's Arch. 142: (series 14, vol. 2) 86–100, 1895.

(31) FELDT, A.: Zur Diagnose maligner Brusthöhlengeschwülste intra vitam. Dt. med. Wschr. 28: 497–499, 1903.

(32) CHACE, A. F.: Diagnosis of carcinoma of the stomach from a fragment of the tumor obtained by means of the stomach tube. Post-Graduate 22: 907–910, 1907.

(33) RICHARDSON, H. L., QUEEN, F. B., and BISHOP, F. H.: Cytohistologic diagnosis of material aspirated from stomach. Am. J. Clin. Path. 19: 328–340, 1949.

(34) GLADSTONE, S. A.: Use of sponge in obtaining tissue from stomach for biopsy. Am. J. Clin. Path. 19: 891–894, 1949.

(35) PANICO, F. G., PAPANICOLAOU, G. N., and COOPER, W. A.: Abrasive balloon for exfoliation of gastric cancer cells. J. A. M. A. 143: 1308–1311, 1950.

(36) PFAHLER, G.: *In* BRYAN, L.: Roentgenological study of primary lung carcinomata. J. Roentg. 2: 10–11, 1921.

(37) NUSSBAUM, R.: Zur Diagnostik des Lungenkrebses. Münch. med. Wschr. 69: 507–508, 1922.

(38) BARKER, L. F.: Carcinoma of the left bronchus. Clin. Med. I, Tuesday Clinics at Johns Hopkins Hospital: 1–32, Philadelphia, 1922.

(39) JEKEL, L. G.: Diagnosis of carcinoma of the stomach from a fragment of the tumor obtained during routine gastric analysis. J. Lab. & Clin. Med. 21: 836–838, 1936.

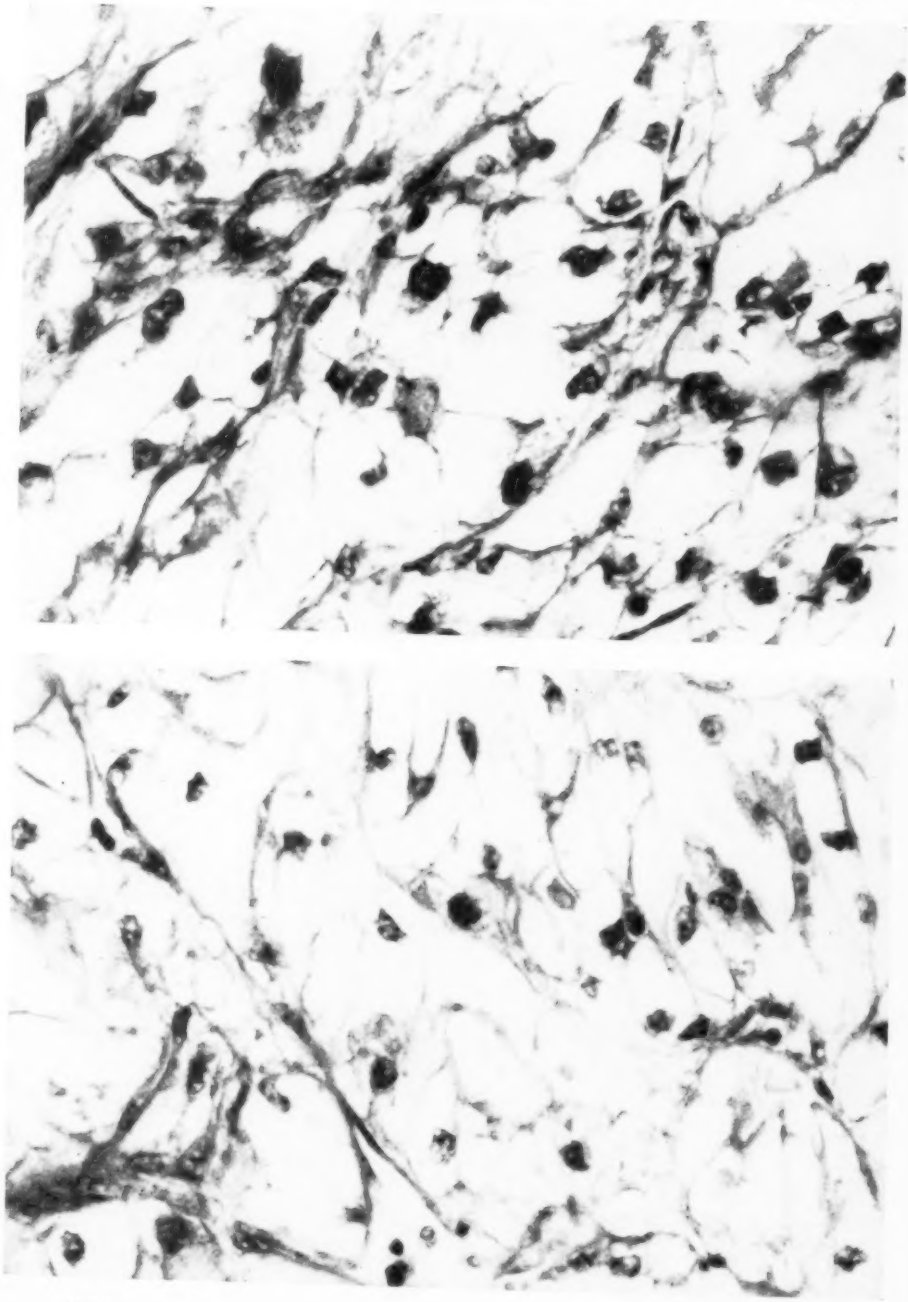
(40) WANDALL, H. H.: A study on neoplastic cells in sputum as a contribution to the diagnosis of primary lung cancer. Act. chir. Scand. 19: (supplement 93) 1–143, 1944.

(41) ADAMS, G.: The cytologic method of cancer detection: a review. Mil. Surgeon 103: 341–344, 1948.

PLATE 110

FIGURE 1.—Upper. Spontaneous biopsy of pulmonary metastasis S–14953, U. S. Marine Hospital, New Orleans, La. × 450.

FIGURE 2.—Lower. Tumor of kidney, S–8362, U. S. Marine Hospital, New Orleans, La. × 450.



Adams

EFFECTS OF A FILTRABLE, SELF-PROPAGATING CONTAMINANT ON A TRANSPLANTABLE ACUTE LYMPHOID LEUKEMIA IN MICE[1]

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Levaditi and Nicolau in 1922 (*1*) described the effect of pox viruses on experimental rodent epitheliomas. Since that time relatively few investigations have been reported on the influence of viruses on neoplasms. A review has been given recently by Moore (*2*) and it is evident that the results reported concerning specific effects of viruses on various types of tumors have been equivocal.

Studies relating to the effects of extraneous viruses on leukemia in particular have been reported recently by Turner and co-workers (*3*, *4*), who observed prolongation of life in mice bearing a transplantable lymphoid leukemia designated Leukemia 9417, when the mice were infected with brain-adapted vaccine virus. During the course of their experiments it was observed that the leukemia became more acute; after which time no effect on the survival of leukemic mice was elicited, although variations in the strain of vaccinia, dosage, and time of infection were tried. Two transplantable myeloid leukemias were unaffected by vaccinia virus. Sharpless and co-workers (*5*) have observed regression of a transplantable lymphoid leukemia in chickens following superimposed infections by any of the following: Russian Spring-Summer, West Nile, Japanese B. encephalitis, St. Louis encephalitis, and louping-ill virus. No apparent damage to the host was observed from these infections, and chickens that did not develop leukemia following transplantation were usually rendered immune to challenge by homologous tumor.

Only two studies (*6*, *7*) concerning the effects of contaminating, nonbacterial, self-propagating agents on experimental lymphomas of mice have been reported. An infectious agent, studied intensively by Taylor and MacDowell (*6*), produced a nonlethal illness beginning at 7 days. Clinical signs were a sudden loss of weight and inactivity accompanied by a moderate lymphocytosis. There was a decrease in the average interval before death following inoculations of infected leukemic-cell brei. The

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causative agent could be separated from the leukemic cells by serial transfer through host animals rendered immune by a prior infection with cell-free filtrates. In the report by de Bruyn (*7*), a cell-free agent that caused a prononced lymphopenia following transplantation of a lymphosarcoma was described. Taylor and MacDowell described alterations in the organs of the infected animals and the leukemic process. De Bruyn's report gave particular attention to the effects on total and differential leukocyte counts, without any description of morphologic changes.

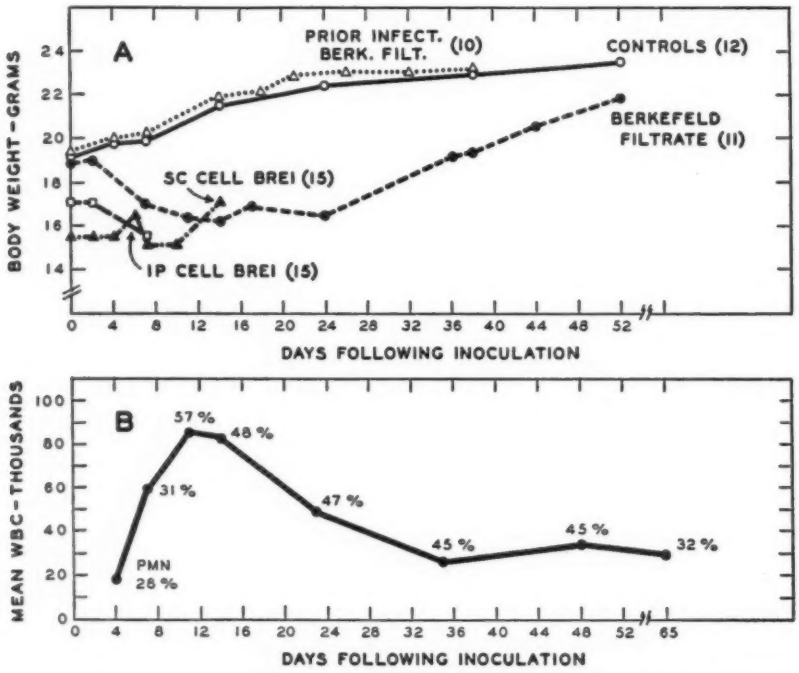
We wish to report studies on the clinical and pathologic effects of a non-bacterial, cell-free, self-propagating agent, probably a virus, which appeared in an acute lymphoid leukemia, L1210, carried routinely in transplant in strain dba mice. Lymphoid leukemia, L1210, has been described previously (*8*). This transplantable leukemia originated in an 8-month-old strain dba female following skin paintings with 0.2 percent methylcholanthrene in ethyl ether. It has been maintained in 4 separate sublines for more than 90 successive passages by transplantation into strain dba mice 4 to 8 weeks of age. In 1 of the transplant sublines, at the 37th passage, all 6 dba animals inoculated in a routine subcutaneous passage of leukemic cells, showed signs of illness 7 days after inoculation. These mice exhibited a pronounced loss in weight, inactivity, ruffled, damp fur, and labored breathing. At autopsy there was cloudy fluid in the peritoneal and thoracic cavities, the spleen, thymus, and lymph nodes were extremely small, and the liver was pale. Signs of the acute illness disappeared in the remaining mice, but they died presumably from leukemia at an average age of 17 days. The extent of infiltration of leukemic cells, however, as determined by histologic study was insignificant when compared with the growth of leukemic cells developing in mice inoculated with the control, uninfected subline, in which massive infiltration into the liver, spleen, and lymph nodes occurred terminally. Propagation of this infected sublime was continued by use of a Locke's-solution brei of homogenized spleen, liver, and lymph nodes inoculated either subcutaneously or intraperitoneally. The infected leukemic cells have now been carried through 26 passages in over 150 strain dba mice and all have exhibited the typical signs of illness. No evidence of this acute infection has been observed in another subline of leukemia L1210 maintained as a control for the infected subline.

An attempt was made at the 4th passage, following discovery of the infection, to separate the contaminant from leukemic cells. Lymph nodes, spleen, and liver were homogenized, diluted 1:10 by volume with sterile Locke's solution, centrifuged at 3,000 r. p. m. for 20 minutes, passed through a tested Berkefeld N candle, then inoculated into 6 strain dba mice intraperitoneally with 0.1 cc. of filtrate per mouse. The filtrate prepared in this manner is referred to as the standard cell-free filtrate. At 7 days the earliest signs of illness appeared, and at 10 days a severe illness with loss of weight was apparent. These mice, however, eventually recovered. Again at the 5th and 6th passages of infected leukemic cells, filtrates were

prepared in identical manner and inoculated into a group of 7 and a group of 10 strain dba mice. All mice showed typical signs of illness, beginning at 7 days, and all eventually recovered. Beginning with the 7th passage of the infected subline, a standard cell-free filtrate, was obtained and used for infecting strain dba mice. Subsequently the infection has been transmitted by standard cell-free filtrate through 22 successive passages using lymph nodes, spleen, and liver from mice inoculated 7 to 21 days previously. In this series 93 mice were used for successive passages of cell-free filtrates and all developed the typical signs of infection. In the first 13 passages, by standard cell-free filtrates, all mice, except those sacrificed for transfer, recovered. However, an increased virulence of the agent has been indicated in transfers 14 through 26. Nine out of 14 animals, not including those sacrificed for transfer or for tissues, in passages 14 through 17, died of an acute infection at from 13 to 43 days. All strain dba animals inoculated in passages 18 through 26, excepting those sacrificed, died at from 9 to 36 days. It was found also that mice of other inbred strains, C58, RIL, C3H, A, and C57 black, when given parenteral inoculations of the standard cell-free filtrate of the 24th passage developed the acute illness and died after approximately the same interval as strain dba mice. However, mice infected with this virulent and invariably fatal process have not been included in these studies, which are concerned only with animals that exhibited the nonlethal illness.

Standard cell-free filtrates used at the 5th and 8th passages were introduced into a horse-meat infusion broth containing 1 percent peptone and 0.5 percent NaCl at pH 7.4, and streaked on a complete nutrient agar medium including the same ingredients, plus 2 percent agar. Incubation for 72 hours was carried out at 37° C. In addition, whole blood obtained under sterile conditions by cardiac puncture from mice showing typical signs of illness in the 3d and 8th passages, respectively, of the cell-free filtrate series, was placed in both tubes and on agar plates and also incubated at 37° C. for 72 hours. No bacterial growth was found in any of the tube or plate samples.

Mice inoculated with cell-free filtrates or with leukemic-cell brei showed a weight loss of at least 10 percent at 7 days (text-fig. 1A). A gradual recovery in body weight commenced after 24 days in the filtrate-inoculated group and continued until the original weights of the animals and more had been regained. The loss in weight was invariably accompanied by ruffling of the fur, inactivity, and labored breathing. Recovery in weight began after complete disappearance of these signs. Periodic blood counts showed an increase in the leukocyte count, concomitant with clinical signs of illness, reaching a maximum of 85,000 at 11 days. This was followed by a long, gradual decline in the leukocyte number and a return to a normal blood count (text-fig. 1B). Differential leukocyte counts revealed an increase in the granulocytes. Severe anisocytosis and polychromatophilia occurred during the period of leukocytosis. At 35 days, postinoculation, the erythrocytes resumed a normal appearance.



TEXT-FIGURE 1.—A. Comparative body weights, at various times intervals, of several groups of experimental and control strain dba mice. SC-cell brei refers to mice inoculated subcutaneously with leukemic cells of the infected subline of leukemia L1210 and IP-cell brei refers to mice inoculated intraperitoneally with leukemic cells. First curve shown represents mice recovered from a prior infection and given Berkefeld filtrate. Compare with curve 3 of mice not previously infected and given Berkefeld filtrate. Control growth curve was determined from uninoculated mice. Points on curves represent means of the number of animals shown in parentheses. B. Periodic leukocyte counts shown in strain dba mice infected with the standard cell-free filtrate. Percentages refer to percent granulocytes obtained from the differential leukocyte counts. Each point represents a mean of 10 mice.

Organ weights obtained at 10 days, at the height of the illness, following inoculation of a standard filtrate, showed an increase in adrenal weights and decrease in weights of the thymus, the spleen, and representative lymph nodes (table 1).

The 2d to 8th passages, inclusive, of the virus-contaminated line, carried by leukemic-cell brei inoculations, were used to determine the effect on survival time of strain dba mice. Leukemic-cell brei (a 0.1 cc. dose of a 1 : 10 dilution in Locke's solution), containing approximately 8×10^5 cells, was inoculated either intraperitoneally or subcutaneously. Similar inoculations of leukemic cells from an apparently uninfected subline of leukemia L1210 were used as control material. It can be seen from table 2 that a statistically significant shortening of the interval before death resulted

TABLE 1.—*Comparative body and organ weights at 10 days of virus-infected and control strain dba mice*

	Body weight (grams)			Spleen (milligrams)	Paired adrenals (milligrams)	Thymus (milligrams)	Paired axillary and brachial lymph nodes (milligrams)	Liver (milligrams)
	Initial	5 days	10 days					
Virus infected	19. 1	17. 5	16. 5	106. 4	5. 8	23. 5	18. 8	900. 8
Controls	18. 9	19. 6	20. 2	247. 8	2. 8	62. 8	39. 3	1, 093. 5
Controls / Infected			1. 23	2. 33	0. 48	2. 67	2. 09	1. 21

Litter mate strain dba male mice weighing 18–20 grams were used. Weights represent the means of 5 animals.
Wet weights to the nearest milligram were obtained on a Roller-Smith microtorsion balance.

TABLE 2.—*Influence on survival time of strain dba mice following inoculations of infected leukemic cells of lymphoid leukemia, L1210*

Experiment	Route of inoc.	Number mice	Death in days																Mean	Standard deviation
			8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
Infected L1210	Subcut	43	----	----	----	4	4	2	7	7	6	1	5	5	----	----	1	1	----	15. 5±0. 45	2. 90
Control L1210	"	42	----	3	3	3	14	8	6	3	2	----	----	----	----	----	----	----	----	12. 5±0. 18	1. 17
			Difference of means = 3.0 ± 0.48; P < 0.001																	

Experiment	Route of inoc.	Number mice	Death in days												Mean	Standard deviation
			5	6	7	8	9	10	11	12	13	14	15	16		
Infected L1210	Intrap	37	----	9	17	7	4	----	----	----	----	----	----	----	7. 1±0. 15	0. 92
Control L1210	"	84	----	----	----	22	15	20	11	9	4	1	2	----	9. 9±0. 19	1. 73
			Difference of means = 2.8 ± 0.24; P < 0.001													

following intraperitoneal inoculations of virus-contaminated leukemic-cell brei, and a statistically significant lengthening of lives of leukemic mice occurred following subcutaneous inoculations. The contaminating agent when inoculated free of leukemic cells, as a filtrate, was found to be nonlethal during this period but inoculated in conjunction with leukemic cells (leukemic-cell brei) it decreased the interval before leukemic death, if inoculated intraperitoneally. Histologic studies revealed that usual extensive infiltrations by leukemic cells found in leukemia L1210 was greatly reduced in mice inoculated subcutaneously, and at the time of death in mice inoculated intraperitoneally, few, if any, leukemic-cell infiltrations were noted.

Studies of resistance to reinfection were carried out by using the standard cell-free filtrate, previously described, and the standard dose of leukemic-cell brei (approximately 8×10^5 cells) from the infected subline. Twelve strain dba mice infected 39 to 48 days previously by inoculation of a standard cell-free filtrate, received a second dose of filtrate. In no case did clinical signs of the infection or of leukocytosis develop. The growth curve of these mice paralleled that of the control series (text-fig. 1A). In 10 control mice inoculated with the same filtrate, typical signs of infection were evident beginning at 7 days. Ten strain dba mice that had been infected previously at from 45 to 123 days, by inoculation of the standard dose of a cell-free filtrate, received a standard dose of leukemic-cell brei from the 4th generation transfer of the infected subline of leukemia L1210. None of these mice showed any signs of illness, all gained in weight, and signs of leukemia were fully manifest after the usual time interval for this transplantable leukemia. Table 3 gives the

TABLE 3.—*Total and differential leukocyte counts at 7 days in strain dba mice following subcutaneous inoculation of leukemic-cell brei of the infected subline, leukemia L1210* [1]

Group	Total wbc	Hb gm. percent	Differential count—percent (absolute number)				
			Lympho-cytes	Poly-morphs.	Mono-cytes	Eosin-ophils	Lympho-blasts
I. Prior infection (5)	48, 130	11. 7	37. 5 (18, 050)	47 (22, 620)	1	------	14. 5 (6, 980)
II. Not previously infected (5).	35, 400	14. 0	28. 5 (11, 860)	66. 5 (23, 540)	5	------	--------
III. Uninoculated controls (5).	13, 700	15. 0	60. 5 (8, 768)	34. 5 (4, 932)	3. 5	1. 5	--------

[1] Strain dba male mice weighing approximately 18 grams were used. No loss in weight was found in group I, previously infected; 20 percent loss in weight found in group II. Figures in parentheses are numbers of mice used for blood counts.

mean total and mean differential leukocyte counts of leukemic mice that had been infected previously by a cell-free filtrate compared with values of dba leukemic mice that had not been infected. It can been seen that at 7 days a typical leukemic blood picture is present in those mice infected previously, whereas there is no evidence of leukemia at this time in the uninfected group. The mean age at death in group I, showing a fully

developed leukemia, was 11.7 days, the expected mean age at death for normal strain dba mice bearing leukemia L1210, while the mean age at death for 5 strain dba mice, not previously infected, and which showed typical signs of illness was 18.3 days.

Since it was found that resistance to infection could be produced in strain dba mice by inoculations of cell-free material, and since these infection-resistant mice developed a fully manifest leukemia following inoculation of leukemic cells from the infected subline of leukemia L1210, an attempt was made to free the infected subline from the contaminating agent. Infected leukemic cells were passed by successive transfers through infection-resistant mice, that is, those previously infected by cell-free material with eventual recovery from the acute disease. Following the 1st passage through a strain dba mouse that had been infected 24 days previously, a terminal leukemic condition, without any evidence of infection, was found at 8 days. These leukemic cells were then passed, using the standard dose of leukemic-cell brei, through 3 successive transfers in host animals that had recovered from previous infections. Following the 1st passage, a test for the presence of the virus was made in 3 normal dba mice. All developed the typical signs of illness. However, after 3 successive passages in previously infected hosts, the transplanted leukemic cells were then apparently free of the infectious agent. Eleven consecutive transfers of leukemic-cell brei in normal strain dba mice have now been accomplished without appearance of the signs of infection.

Histologic examinations were made of several groups of these animals; 1) mice bearing leukemia L1210 before the virus infection occurred, 2) mice infected with the virus, but without leukemic cells, 3) mice transplanted with the leukemia infected by the virus, and 4) mice that had recovered from the virus infection several months previously. These groups are discussed below.

1) This group consisted of the mouse in which Leukemia L1210 originated, and four other animals transplanted with this line before the virus infection was recognized. Since this transplantable leukemia has been carried for a long time in this laboratory and has been used previously in a study of the effects of aminopterin (*8*), its morphology and biologic behavior have been closely observed. It is considered to be a lymphoid leukemia. The principal cell is large and mononucleated, with a large vesicular nucleus and prominent nucleoli, a distinct nuclear membrane, and a narrow rim of basophilic cytoplasm. There is little variation in the size or morphology of the cells, and mitotic figures are frequent. At the time of death the leukemic cells form a large, localized mass at the site of subcutaneous transplantation, and numerous cells are dispersed in the lymph nodes and spleen, enlarging these organs and obscuring the architecture. The leukemic cells appear in the peripheral blood a few days after transplantation, and very high leukocyte counts develop before death. In the original mouse, the lymph nodes and spleen were greatly enlarged and uniformly infiltrated by the leukemic cells. The four animals that had early transplant generations of leukemia L1210 showed this

same picture, with infiltration of the spleen, lymph nodes, and liver by the leukemic cells. Two of these animals also showed a lesion in the lung, which we have occasionally observed in leukemia in mice. It consists of necrotic cells and masses of fibrinoid material contained in a pulmonary artery (fig. 1).

An additional animal, which should be considered here, had recovered from the virus infection several weeks before it was transplanted with leukemia L1210. It showed a fully developed leukemia characteristic of this line and no morphologic evidence of infection by the virus.

2) This group consisted of five mice that had received the standard cell-free filtrate 10 to 14 days previously. These mice showed clinical signs of acute infection. The pathologic findings were essentially the same in each of these five animals, so they are described together. All showed ascites and considerable subcutaneous edema, and two had hydrothorax. The livers were generally pale, and the thymus, the spleen, and the lymph nodes were greatly reduced in size. Some nodes appeared hemorrhagic, and several showed minute yellowish-white opaque areas. Similar opaque areas were seen in the subcutaneous areolar tissue, and in the adipose tissue surrounding the reproductive organs, and in the mesentery. The microscopic observations were:

Lymph nodes.—The most outstanding change in the lymph nodes was the almost complete absence of lymphocytes (fig. 2). No follicles could be recognized in the most severely affected nodes, which consisted of a reticular framework, reticulum cells, and wide sinus spaces. In some nodes there was a pronounced infiltration of plasma cells, some of which contained Russell bodies. (Russell-body cells were noted by Taylor and MacDowell as common in the virus infection described by them.) A scattering of granulocytes was occasionally found, more commonly in the hilus and often intermingled with the plasma cells. The reticulum cells often appeared increased in number, but this was probably relative, due to the disappearance of lymphocytes. A fibrinoid degeneration of the supporting reticular framework often appeared in the node, and a similar type of degeneration often affected the capsule of the node and the walls of nearby vessels.

Lesions in the surrounding areolar and adipose tissue of the nodes, in the subcutaneous tissue, and in the genital omentum and mesentery were similar and are described together. Minute areas of necrosis, which were not sharply defined and centrally involved all tissue elements, were surrounded by an infiltration of plasma cells, monocytes, and occasional granulocytes (fig. 3). Accumulations of similar cells often produced a distinct "cuffing" about small vessels, both arteries and veins. Occasionally the vessel wall showed fibrinoid degeneration, as mentioned above.

Spleen.—The spleen generally showed a less severe reduction in lymphocytes than did the lymph nodes and thymus, so that the Malpighian bodies were distinguishable. A moderate degree of hematopoiesis was present in the pulp. The megakaryocytes appeared normal. The most distinctive lesion was fibrinoid necrosis of the Malpighian bodies, affecting

the outer portion more severely, and usually sparing the central arteries and the immediately adjacent tissue (fig. 4). The lesion sometimes involved only a few bodies in a section. The necrosis often extended to the adjacent pulp, and the capsule was depressed above the lesion. In one severely affected spleen, however, every follicle in the section was involved in this necrotic process.

Thymus.—The thymus was also seriously affected. As in the lymph nodes, there was an almost complete absence of lymphocytes, so that the cortex was greatly reduced in thickness and consisted only of reticulum cells. The lymphocytes were relatively more abundant in the medulla than in the cortex, a reversal of the normal condition (figs. 5 and 6), such as may be seen in later stages of the "alarm reaction."

Bone marrow.—The bone marrow of the sternum appeared nearly normal in three mice. In two there was considerable hypoplasia, affecting the erythropoietic elements especially (fig. 7).

Liver.—The liver appeared normal in two animals. In the other three the hepatic cells were vacuolated. No fat stains were done.

Adrenal gland.—No distinct alterations were found in the microscopic sections from the adrenal gland except for a slight dilatation of vessels of the medulla.

Lung.—The alveolar walls were congested, and there appeared to be an increased number of leukocytes in the interstitial tissue. This increase of cells in the interstitial tissue probably reflected the elevated leukocyte count of the peripheral blood. Very few leukocytes were present in the alveolar spaces, there was no pneumonic consolidation, and the lesions in the lungs were considered to be minimal.

Other organs.—No significant changes were found in other organs of these virus-infected animals. Apparently this process affected the lymphocytes and the lymphatic system selectively. A search was made to determine whether the thrombotic lesion in the vessels of the lung was restricted to animals with leukemia or whether it was evidence of the virus infection. No thrombosis of pulmonary vessels was found in the present group of animals, so it seems probable that this lesion was directly associated with this leukemic strain.

3) This group consisted of 10 animals receiving a subcutaneous transplant of leukemic cells infected by the agent. Seven showed severe effects of the virus, with all the lesions described in the preceding group. In these animals, little or no evidence of leukemia was found. In 1 case especially, only a few cells, leukemic in type, were found in the connective tissue of the mesentery (fig. 8) but all other organs and tissues were free of such cells. On the other hand, the 3 mice that showed least evidence of infection had definite leukemic infiltration in the lymph nodes and spleen. Necrotic material in the pulmonary artery was present in 1 animal of the 1st group, and 1 lymph node in this group had a great number of Russell-body cells. This was the only node examined in which these cells were strikingly increased.

4) This group consisted of five animals examined 5 months after infection with the virus. After the severe injury to the lymphatic tissue was observed in the acute stage of the virus infection, the question was raised as to how complete the recovery from such a process might be. Mice recovered from the infection were, therefore, examined. No changes were found in the lymph nodes, spleen, or thymus that could be ascribed directly to the preceding infection, except that one spleen contained a small area of fibrosis just below the capsule. Three mice had very small local subcutaneous abscesses in the flank. These same three mice showed moderate amyloidosis of the spleen. The lymph nodes showed slight hyperplasia of reticulum cells, and the involution of the thymus was more advanced than was usual for this age group. Occasional masses of lymphocytes, and perivascular lymphocytic accumulations were present in the lungs, liver, and kidney. Three animals had calcification of the myocardial surface, such as is frequently found in strain dba mice. Altogether the mice in this group were not significantly different from strain dba mice of the same age, and there was no lesion pathognomonic of the preceding infection.

DISCUSSION

The conclusion that a self-reproducing, nonbacterial, virus-like agent is responsible for the changes observed in this particular subline of acute lymphoid leukemia is supported by several observations. Evidence was obtained from experiments on serial transfers that there existed a contaminating agent in cell-free filtrates obtained from the transplantable leukemic line, and no growth of bacteria in cultures of cell-free filtrates and blood from infected animals could be found. It is possible that the virus is carried in a latent condition in strain dba mice and was activated in rapidly growing leukemic tissue carried in transplant. This seems unlikely, however, since at least one other subline of the same leukemia, L1210, used here as control material, and two other transplantable lymphomas carried by transplantation in strain dba mice have apparently remained infection-free. Furthermore, it seems that the virus is not present in the breeding colony of strain dba mice, since all the mice of this strain inoculated with infected leukemic-cell brei or infected with cell-free filtrates failed to resist the infection.

Although a statistically significant lengthening of life of leukemic mice bearing subcutaneous implants of the infected subline was obtained, death apparently resulted from the combined effects of leukemic cells and virus, although virus alone was nonlethal. It appears that a more striking increase in survival time would be expected using a less concentrated inoculum of virus because of the severe damage to lymphoid tissue. Titration experiments have not been attempted in this study.

There is no evidence from histologic studies of a specific tropism of the virus-like agent described here for leukemic as opposed to normal cells of the lymphocytic series or other types of neoplastic cells. It may be expected, however, that any neoplasm exhibiting extremely rapid growth

would support proliferation of virus most successfully and in consequence influence the growth of the neoplasm.

Certain striking differences exist between the effects produced by the contaminating agent described here and the agent found by Taylor and MacDowell in a strain C58 transplantable leukemia. The leukocytosis that invariably accompanied the acute illness was granulocytic in type in contrast to a lymphocytosis observed in the strain C58 leukemia. Severe lymphatic atrophy was characteristic of our line L1210, whereas lymphatic hyperplasia accompanied the acute infection in line I of Taylor and MacDowell. Fibrinoid degeneration of the reticular framework of lymph nodes and the surrounding areolar and adipose tissue and of the outer portion of the Malpighian bodies of the spleen was found in our mice in the acute stages of infection. The only distinct lesions mentioned by Taylor and MacDowell were "small lymphoid lesions, both perivascular and scattered through the sinusoids" of the liver. Thus it would appear that the agents found in the dba and C58 strains are not analogous. Work is now in progress at this laboratory to identify the agent described here.

It has been noted that in carrying tumors by transplantation for prolonged periods of time, the tumor lines do not always behave uniformly (*9*). The reasons for the variable behavior cannot always be explained adequately. Most tumor lines have been reported to increase in virulence after transplantation, exhibiting a faster growth rate and a reduced survival time. This change is sometimes, but not always, correlated with a recognizable morphologic alteration. When the morphologic change is recognizable, it would seem that a change in the tumor results from selection of cells used for transplantation. When a morphologic change is not evident, it is probable that spontaneous mutations of so-called histocompatability genes influence the behavior of the tumor (*10*). It remains a distinct possibility that changes in the behavior of a transplantable tumor, and its effect on the host, may be due to an extrinsic agent transmitted with the tumor. Such agents have been infrequently recognized but are apparently more common than suspected as shown by Taylor and MacDowell in a survey of several transplantable lymphomas (*6*). In the present study the biologic behavior of a transplantable lymphoid leukemia was changed by a virus-like agent. The morphologic appearance of the tumor cells, however, was unchanged. In this instance the presence of the agent was recognized easily because of its severe influence on the host. Other contaminating agents, however, may be more difficult to recognize.

The fact that the contaminating virus so seriously injured the normal lymphocyte and influenced the growth and infiltration of the neoplastic lymphocyte invites speculation about the usefulness of this approach in the therapy of leukemia. Suggestive evidence has been presented by Sharpless (*5*), Turner (*3*, *4*), and Bierman (*11*) that viral agents may affect leukemia, probably favorably. There is no indication, however, that the viruses reported as influencing favorably the course of leukemia have any other properties in common, and most are impractical for use in clinical medicine.

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SUMMARY

A subline of a transplantable acute lymphoid leukemia in strain dba mice has been shown to carry a filtrable, self-propagating, virus-like agent. In the early transfer generations the virus produced in strain dba mice a nonlethal illness characterized by loss in weight, inactivity, ruffling of fur, and labored breathing beginning at 7 days. Accompanying these signs of illness was a moderate leukocytosis, due principally to an increase of granulocytes.

Intraperitoneal inoculations of virus-infected leukemic cells reduced the mean age at death from leukemia, while inoculations subcutaneously increased the mean age at death.

The virus has been separated from leukemic cells by filtration, and has been maintained by serial transfer of cell-free filtrates in dba mice, calling forth regularly the typical signs of acute illness.

Reinfection of recovered animals has been resisted regularly. By passing the infected subline through successive hosts that have recovered from the illness, the leukemic cells were then apparently freed of the virus.

In later transfer generations of the virus a change in virulence has been noted resulting in death of the majority of inoculated animals.

Extreme reduction of the number of lymphocytes in the lymph nodes, thymus, and spleen was observed on histologic examination at the height of the infection. Hyaline necrosis in the lymph nodes, spleen, and adipose connective tissue was found. Animals inoculated with leukemic cells and virus showed remarkably little microscopic evidence of leukemia.

REFERENCES

(*1*) Levaditi, C., and Nicolau, S.: Sur la culture de virus vaccinal dans les neoplasmes epitheliaux. Compt. rend. Soc. de biol. 86: 928–939, 1922.

(*2*) Moore, A. E.: Effect of inoculation of the viruses of influenza A and Herpes simplex on the growth of transplantable tumors in mice. Cancer 2: 516–524, 1949.

(*3*) Turner, J. C., Mulliken, B., and Kritzler, R. A.: Influence of vaccine virus on a transmissible leukemia of mice. Proc. Soc. Exper. Biol. & Med. 69: 304–306, 1948.

(*4*) Turner, J. C., and Mulliken, B.: Effects of intravenous vaccinia in mice with sarcoma 180 or leukemia 9417. Cancer 3: 354–360, 1950.

(*5*) Sharpless, G. R., Davies, M. C., and Cox, H. R.: Antagonistic action of certain neurotropic viruses toward a lymphoid tumor in chickens with resulting immunity. Proc. Soc. Exper. Biol. & Med. 73: 270–275, 1950.

(*6*) Taylor, M. J., and MacDowell, E. C.: Mouse leukemia. XIV. Freeing transplanted Line I from a contaminating virus. Cancer Research 9: 144–149, 1949.

(*7*) De Bruyn, W. M.: A lymphopenia-causing agent, probably a virus, found in mice after injection with tumor tissue and with cell-free filtrates of Lymphosarcoma T–86157 (MB). Cancer Research 9: 395–397, 1949.

(*8*) Law, L. W., Dunn, T. B., Boyle, P. J., and Miller, J. H.: Observations on the effect of a folic-acid antagonist on transplantable lymphoid leukemias in mice. J. Nat. Cancer Inst. 10: 179–192, 1949.

(*9*) MacDowell, E. C.: Variation in leukemic cells of mice. Symposia on Quant. Biol. XI: 156–176, 1946.

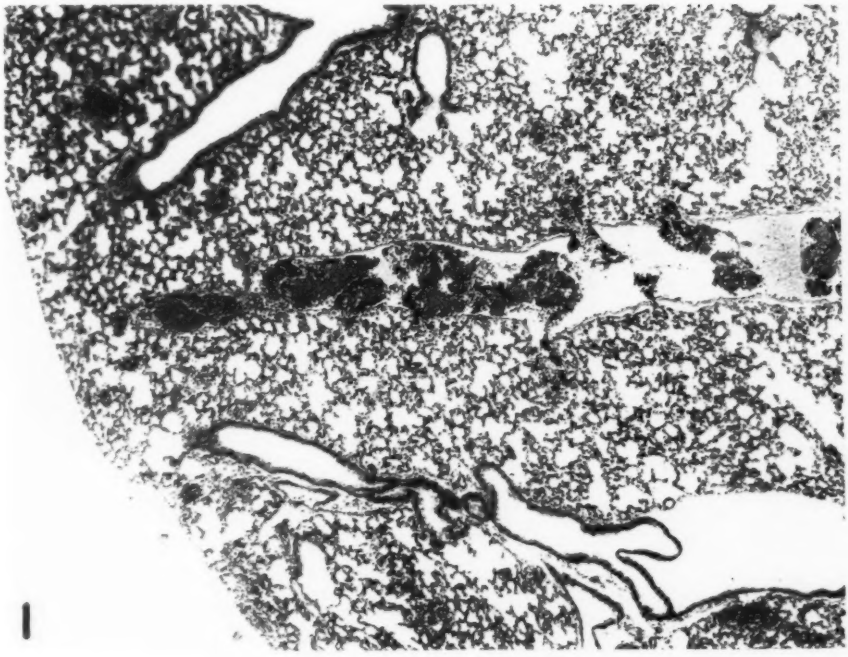
(*10*) Snell, G. D.: Methods for the study of histocompatibility genes. J. Genetics 49: 87–108, 1948.

(*11*) Biebman, H. R., Hammon, W. M., Eddie, B. U., Meyer, K. F., and Shimkin, M. B.: The effect of virus and bacterial infections on neoplastic diseases. Abstract. Cancer Research 10: 203–204, 1950.

PLATE 111

FIGURE 1. Lung of animal with leukemia L1210. Note thrombosis in a pulmonary artery. Hematoxylin and eosin stain. × 60.

FIGURE 2.—Lymph node from mouse with virus infection. Note absence of follicles, reduced number of cells, and empty sinuses. Collections of plasma cells are present in the hilum and around large artery. Hematoxylin and eosin stain. × 110.



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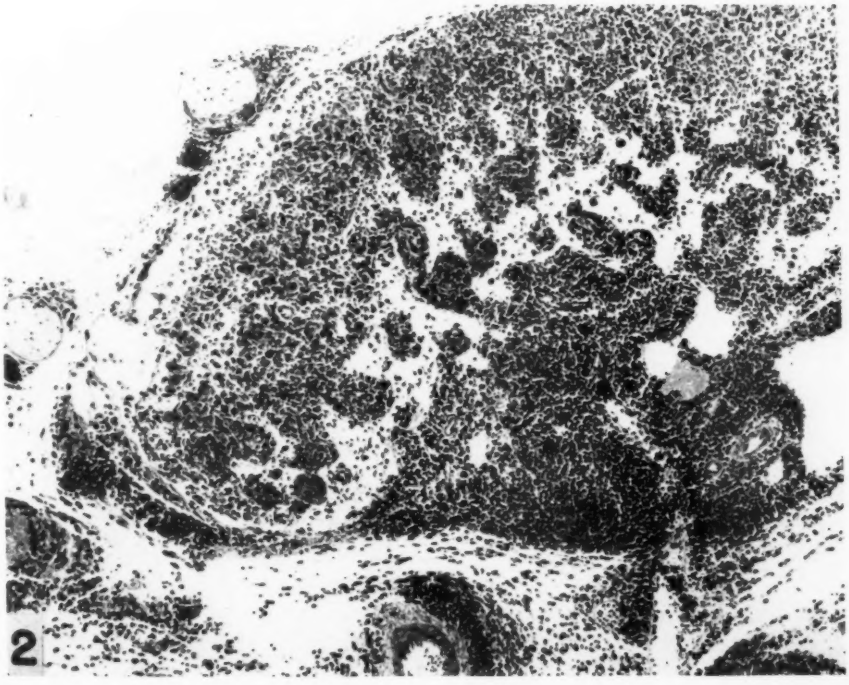

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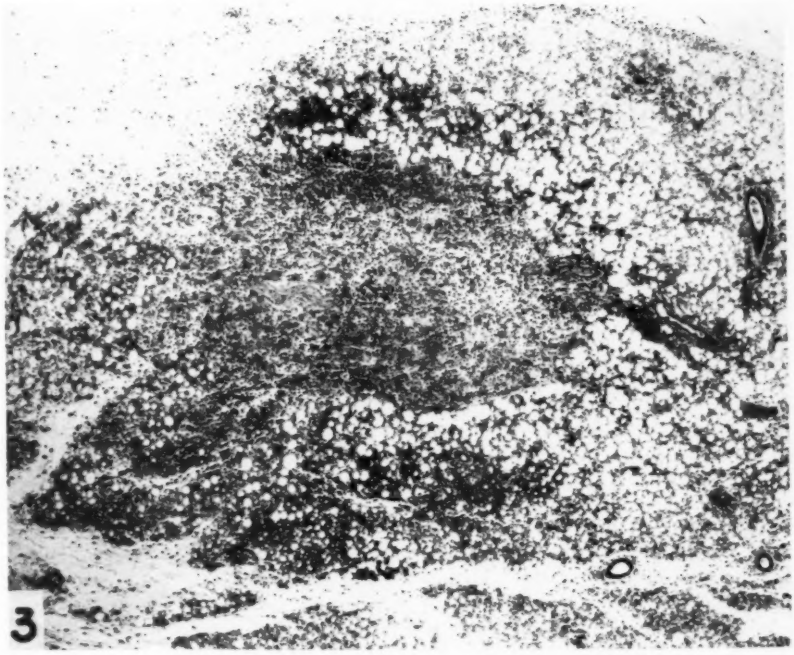
PLATE 112

FIGURE 3.—Subcutaneous areolar tissue from mouse with virus infection. Note focal area of necrosis and infiltration of leukocytes in surrounding tissue. Hematoxylin and eosin stain. × 55.

FIGURE 4.—Spleen from mouse with virus infection. Note necrosis affecting Malpighian bodies and adjacent pulp, with depression of overlying capsule. Hematoxylin and eosin stain. × 50.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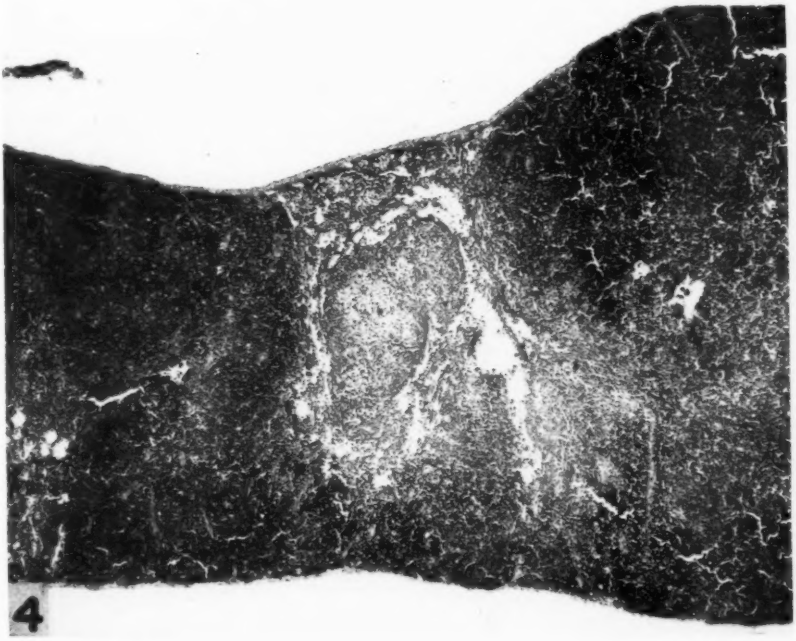
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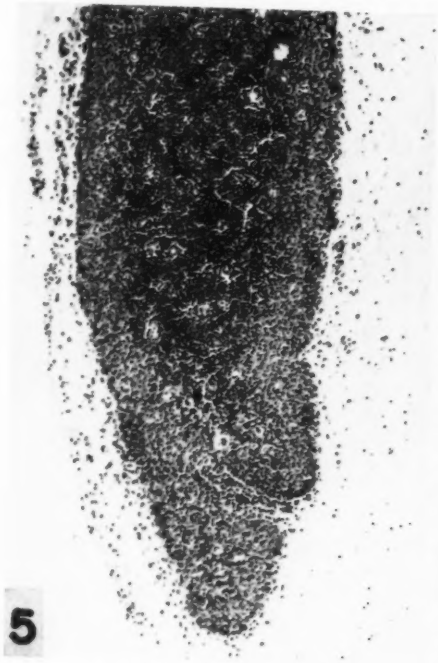
PLATE 113

FIGURES 5 and 6.—Thymus of a mouse with virus infection (fig. 5) compared with thymus of a normal mouse (fig. 6). Note great reduction in width of cortex and loss of lymphocytes. More lymphocytes are found in the medulla. Hematoxylin and eosin stain. × 110.

FIGURE 7.—Bone marrow from mouse with virus infection. Note reduction in number of cells. Hematoxylin and eosin stain. × 250.

FIGURE 8.—Leukemia L1210 and virus infection. Leukemic cells, many of which are necrotic, are present in fat tissue in the mesentery. Such lesions as this were the only evidence of leukemia in some of these animals. Hematoxylin and eosin stain. × 380.



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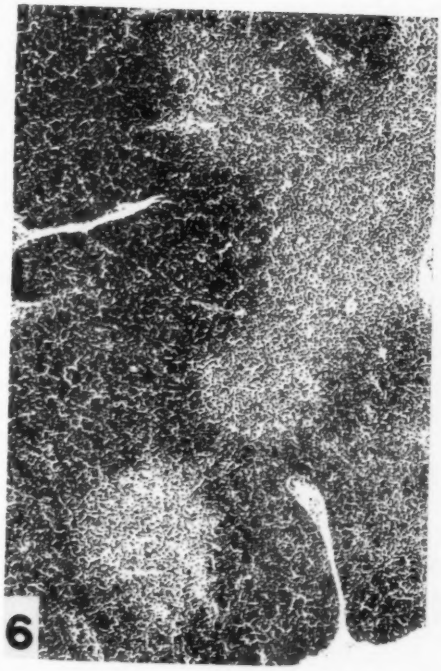

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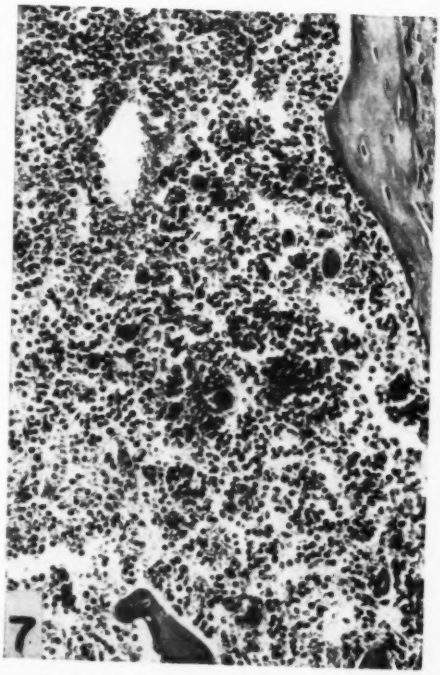

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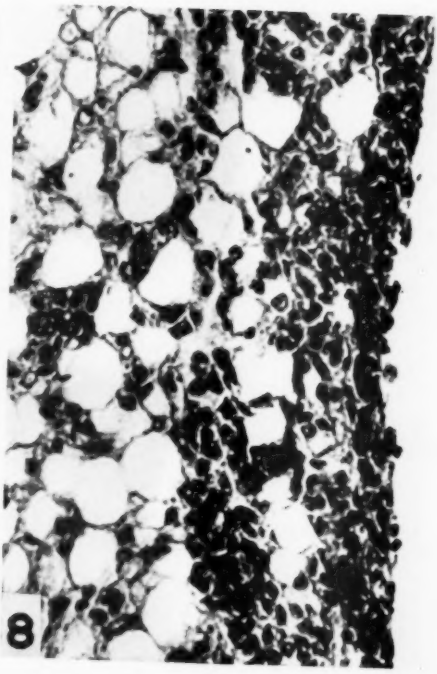

8

TUMOR DEVELOPMENT IN SUSCEPTIBLE STRAIN A AND RESISTANT STRAIN L LUNG TRANSPLANTS IN LA F_1 HOSTS [1]

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The influence of genetic factors on tumor development has been demonstrated as clearly in pulmonary tumors of mice as in any type of tumor (*1*, *2*). Even specific genes have been shown to affect the incidence of tumors and the number of tumor nodules occurring in the lungs of each animal (*3*, *4*, *5*). Despite the clear demonstration of the influence of genes, very little has been learned concerning the physiologic mechanisms through which the action of the genes becomes manifest. It has not even been known whether the genic action was localized in the pulmonary tissue or was manifested through some systemic physiologic mechanism indirectly effecting the response of the pulmonary cell. Furthermore, little has been learned regarding the physiology of pulmonary-tumor development in mice that would lead to the answer of the question. Following the discovery of the important role of hormones in the development of tumors of the mammary glands in mice, logical steps of experimentation revealed that the action of certain genes controlling mammary-tumor development was not limited to the mammary-tissue cell but was manifested through the physiology of hormone production.

An approach to the discovery of the site of action of the genes controlling the production of pulmonary tumors was conceived as possible through the transplantation of lung tissues from two inbred strains differing in genetic susceptibility into a common host where systemic influences would be equal. This is possible when the host is the F_1 hybrid of the two inbred strains. If the genic action were localized in the pulmonary tissue itself, tumor development in the transplants should be governed by the genotype of the transplants. If the action were systemic, tumor development of the transplants from the two different sources should be governed by the genotype of the host.

Data presented herein indicate that at least most of the genic action is localized in the lung tissue itself.

STRAINS OF MICE

The 2 strains of mice used were strain A, which is highly susceptible to the development of pulmonary tumors, and strain L (C57 leaden),

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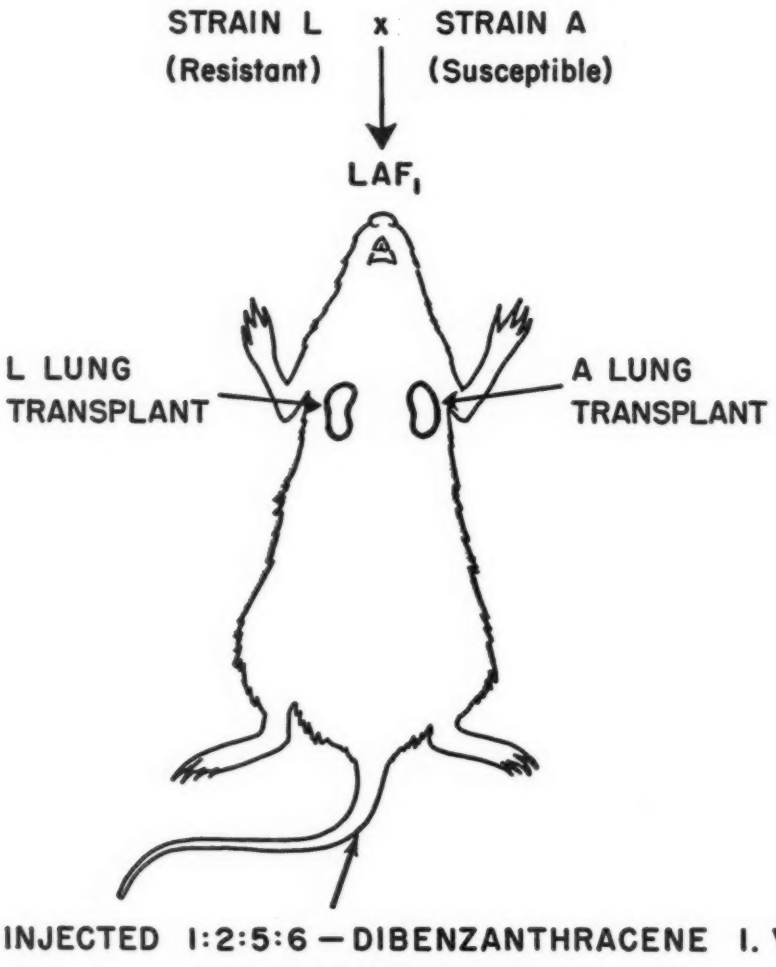
which is highly resistant to the formation of these tumors. These strains have been employed extensively in this laboratory in the analysis of the inheritance of the pulmonary tumor (*1–5*). In this former work, it was found that in strain A mice the incidence of spontaneous pulmonary tumors was approximately 50 percent at 12 months of age and approxi-



TEXT-FIGURE 1.—Outline of experiment.

mately 90 percent at 18 months of age. Following the intravenous injection of 0.5 mg. of 1,2,5,6-dibenzanthracene into mice of strain A an incidence of 100 percent was reached in 6 weeks, and by 16 weeks there was a mean of 75 tumor nodules in the lungs of each individual.

Strain L mice are extremely resistant to the development of pulmonary tumors. Only 3 spontaneous tumors of this type have been observed in the

history of the strain. In previous studies (*1*), intravenous injection of 0.5 mg. of 1,2,5,6-dibenzanthracene into mice of this strain resulted in an incidence of only 24 percent of pulmonary tumors and a mean of 0.27 tumor nodule at 48 weeks following the injection.

EXPERIMENTAL PROCEDURE

For hosts of the transplants, 50 male and 42 female LA F_1 hybrids were secured by mating strain L females with strain A males. At 4 weeks of age, these hybrids were weaned, individually identified, and segregated as to sex in plastic cages with 8 or 9 mice in each cage. Throughout the experiment, they were given tap water and pelleted food *ad libitum*. The formula of the food has been published by Morris (*6*).

At 5 to 6 weeks of age each hybrid mouse received a transplant of a fragment of the lung of a strain A mouse subcutaneously in one axilla and a fragment of the lung of a strain L mouse in the opposite axilla. (text-fig. 1). The lungs of each strain A or strain L donor were cut with fine scissors into about 30 fragments that would fit the 12-gauge trocar used for transplanting the tissue. The donor strain A and strain L mice were the same sex and age as the host hybrid mouse. Approximately 1 month was allowed for the transplants to become established, after which time each host animal was injected in the tail vein with 0.5 mg. of 1,2,5,6-dibenzanthracene dispersed in 0.5 cc. of horse serum.

Twelve to 15 months following injection of the carcinogen, all remaining animals were killed and the transplants recovered. A few mice had been examined earlier, since they were found dead or moribund. The transplants were found to be approximately the same size as they were at the time of implantation, and were identified as distinct masses when the skin was excised. The new blood vessels supplying the transplants served as an aid in identifying them. These transplants of lung tissue were fixed in Tellyesniczky's fluid (70 percent ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part), embedded in paraffin, sectioned serially, and stained with hematoxylin and eosin for histologic examination. At the time of autopsy of the hybrid hosts, the number of nodules in their lungs was recorded, and sections of them were prepared in the same manner for histologic comparison with the transplants.

HISTOLOGY OF LUNG TRANSPLANTS

The primary purpose of this experiment was to determine the number of neoplasms in the transplants of lung tissue from the two different strains, A and L, growing in the F_1 hybrid hosts. However, many of the nonneoplastic reactions developing in this transplanted tissue were of interest. Severe inflammatory reactions with abscesses and extensive necrosis were rare, but many of the transplants, particularly those of strain L origin, showed a process resembling bronchiectasis, with dilatation of the bronchi, purulent exudate in the lumen and leukocytic infiltration of the wall. In many cases the alveolar tissue had undergone atrophy

and gave the appearance of loose connective tissue demarcated by the pleural membrane. In other transplants the alveolar spaces were collapsed. Occasionally the transplants were so heavily infiltrated by lymphocytes that they resembled lymphoid tissue. It was concluded that this reaction probably represented a proliferation of the lymphoid elements normally present in the lung tissue, and not necessarily an inflammatory reaction. Large macrophages filled with brown pigment were frequent within the transplant. These macrophages were thought to have arisen from the transplant and not by infiltration from without.

The two lesions of importance in the present study were 1) the pulmonary tumor and 2) areas of proliferation of the bronchial epithelium.

TABLE 1.—*Characteristics by which pulmonary tumors and proliferation of bronchial epithelium were distinguished*

Points of comparison	Pulmonary tumor	Proliferation of bronchial epithelium
Location	Generally at periphery of transplant.	Generally central.
Relation to bronchus.	Usually no connection with bronchus. Occasionally a secondary extension into bronchus.	Generally connected with bronchus, as determined by serial sections.
Extent	Rarely multiple. Some of comparatively large size, and of greater extent than original transplant.	Involving single or many areas, often diffuse.
Shape	Rounded.	Often fan-shaped, spreading out from a large bronchus.
Margin	Compressed tissue, with partial capsule.	No encapsulation.
Inflammatory reaction.	Variable. Often absent.	Often considerable in lung and surrounding tissue.
Cellular structure	Identical in structure with usual pulmonary tumor. Cords of uniform cells, with cleftlike spaces.	Often columnar epithelium. Basal location of nuclei. Frequent ciliated cells, surrounding circular spaces, containing granulocytes and macrophages.

The pulmonary tumor developing in this material is identical with that occurring spontaneously in mice. It has a distinctive morphology. In order to distinguish the pulmonary tumor as accurately as possible from the proliferative lesions of the bronchial epithelium, a table has been prepared to contrast the characteristics by which each lesion was diagnosed (table 1). In contrast to the pulmonary tumor, the proliferative lesion of the bronchial epithelium was a less distinctive, more diffuse process, with gradations from a small focus to large areas composed of cells similar to those of the bronchial epithelium. Nevertheless, a tabulation of these foci of epithelial proliferation seemed justified, since serial sections were studied. The presence or absence of the process, and not the individual foci, was tabulated (table 2).

A series of photomicrographs (figs. 1–6) depicts certain histologic features of the pulmonary tumors and the proliferation of bronchial epithelium.

The histologic features of the proliferation of the bronchial epithelium were similar to those found in the guinea pig (*7*) and in the rat (*8*) in association with chronic inflammation. The lesion in the transplanted lung tissue was proliferative and not neoplastic. The participating epithelial cells resembled those of normal bronchial mucosa, and often possessed cilia. This lesion also resembled a lesion found in strain dba mice (*9*) usually associated with a bronchiole or alveolar duct, and considered to be bronchial dilatation and proliferation associated with inflam-

TABLE 2.—*Occurrence of tumors and proliferation of bronchial epithelium in lung transplants*

Hosts			Transplants			
Time in months after injection	Number of animals	Sex	Strain of donor	Number of transplants	Number with tumor	Number with proliferation of bronchial epithelium
7	1	♂	A	1	0	0
			L	1	0	1
8	2	♂	A	2	0	0
			L	1	0	1
10	1	♂	A	1	1	0
			L	1	0	0
11	1	♂	A	1	0	1
			L	1	0	0
12	34	♂	A	34	15	1
			L	32	0	7
13	2	♂	A	2	1	0
			L	2	0	1
14	9	♂	A	7	0	4
			L	9	0	7
4	1	♀	A	1	1	0
			L	1	0	0
10	2	♀	A	1	0	1
			L	2	0	2
11	2	♀	A	2	2	0
			L	2	0	2
12	26	♀	A	26	10	2
			L	23	2	8
13	3	♀	A	3	0	0
			L	3	1	1
14	6	♀	A	4	2	1
			L	5	0	3
15	2	♀	A	2	2	0
			L	1	0	1
Total	92	♂ & ♀	A	87	34	10
			L	84	3	34

mation. In the mouse the distinction between bronchi and bronchioles is difficult to make even in the intact lung. According to Fekete (*10*), the bronchial tubes in the lung have no cartilage in their walls, and the bronchiolar epithelium is not ciliated. In the transplanted lung fragments, no differentiation between the bronchi and bronchioles was attempted. Since ciliated epithelial cells were frequent in the proliferative lesion, it seems certain that they were partly, if not entirely, derived from the bronchi.

When the two distinctive lesions, namely, the lung tumor and the focus of proliferation of bronchial epithelium, were characteristically developed, there was no difficulty in distinguishing between them. Occasionally

lesions were encountered, however, in which the diagnosis was uncertain. These have been designated as questionable lung tumor. Although they had the cellular morphology of the pulmonary tumor, such lesions were small in size, they were sometimes centrally located, and microscopic details were obscured by adjacent areas of proliferation of bronchial epithelium and inflammatory reaction.

INCIDENCE OF TUMORS IN THE TRANSPLANTS OF LUNG TISSUE

The occurrence of tumors and the proliferation of bronchial epithelium in the transplants are given in table 2. From the 50 LA F_1 host males, 48 strain A and 47 strain L transplants were recovered. Typical pulmonary tumors were found in 17 of the strain A transplants and in none of the strain L transplants. From the 42 LA F_1 females, 39 strain A and 37 strain L transplants were recovered, and typical pulmonary tumors were found in 17 of these strain A transplants and in 3 of the strain L transplants. There was not a significant sex difference. Typical pulmonary tumors occurred in 39.1 percent of the strain A transplants and in 3.6 percent of the strain L transplants, considering the total number of transplants from both sexes. Thus, much of the genetic differences in susceptibility of the two strains was revealed in the tumor incidence of the transplants surviving in the common LA F_1 host.

Questionable lung tumors were observed in two transplants from strain A donors and in one transplant from a strain L donor. In addition, a small nodule arising at the location of one strain A transplant was composed of an aggregation of mast cells. These cells contained varying numbers of granules which stained metachromatically with toluidine blue. No lung tissue was recognized among the mast cells at the site of the transplant. This mast-cell neoplasm may have arisen independently of the transplanted tissue. This lesion was similar to other mast-cell neoplasms described in mice by Deringer and Dunn (*11*). It is of interest to note that the tumors reported by these authors were found in old hybrids of crosses between strains A and L mice. Another strain A transplant showed a small sarcoma. This was particularly interesting in view of the fact that some lung tumors upon metastasizing develop sarcomatous areas, and that some have transformed to sarcoma on serial transplantation (*12*). The transplanted tissue from all animals was remarkably free from squamous-cell metaplasia. Only one section showed keratin in a bronchial lumen.

In table 2 it will be noted that the bronchial epithelial proliferation described in the preceding section occurred in both strain A and strain L transplants. It is noteworthy that it occurred more frequently in transplants from strain L, the strain resistant to lung tumors, than in transplants from strain A donors.

As could be expected from earlier results (*1*), multiple tumors occurred in the intact lungs of all the LA F_1 host mice. The number of nodules ranged from 19 to 60 per mouse, and they appeared as typical pulmonary tumors of this species. In addition, 1 LA F_1 female had an hemangioendo-

thelioma in the posterior region of the abdominal cavity and 1 male had a subcutaneous fibrosarcoma.

DISCUSSION

That pulmonary tumors can develop in transplanted lung tissue is an established fact. In an attempt to develop a better test for carcinogenesis through homologous transplants of minced fetal tissues, Smith (*13*) found typical lung tumors in transplants of strain C fetal lung injected along with dibenzanthracene or methylcholanthrene into the thigh muscle. Horning (*14*) has reported tumors of bronchial origin in homologous lung transplants treated with crystals of carcinogens at the time of transplantation. Without studying his histologic sections, it is difficult to ascertain how his material compares with that we have described. The photographs suggest a process of proliferation of bronchial epithelium such as we observed. In the work reported herein, typical pulmonary tumors arose in fragments of adult lungs transplanted subcutaneously into mice that later were injected intravenously with a carcinogen.

It is tentatively assumed that the carcinogen aided the appearance of these tumors. If this assumption is incorrect, then it must be accepted that the transplantation alone enhanced their development, since more tumors arose in the transplants than would be expected to arise spontaneously in comparable-sized fragments of the lungs of mice of these strains not exposed to a carcinogen.

Since tumors also occurred in the lungs of the hosts, the possibility that those in the transplants were metastases must be considered. This seems highly improbable, however, in view of the fact that pulmonary tumors rarely metastasize, and no metastases have ever been reported to subcutaneous sites. Moreover, although there were many tumor nodules in the lungs of the hosts, they were relatively small, whereas metastases of pulmonary tumors have been observed to develop only from large local growths.

The present study demonstrates how these transplantation procedures can be applied to the analysis of the physiologic genetics of this type of tumor. In addition to the demonstration of genetic effects and linkage studies for which this tumor has proved to be well fitted, an approach is now offered toward the localization of the genic action.

By measuring the difference in degree of susceptibility of strains A and L and by measuring the variance in their F_2 hybrid generation, it has been estimated that the two strains differ by at least four pairs of genes influencing susceptibility to induced pulmonary tumors (*1*). The number is probably greater. Furthermore, an analysis of the incidence of spontaneous pulmonary tumors in the two strains and their hybrids, at definite age intervals, indicated that the two strains likewise differed by multiple genes in respect to spontaneous pulmonary tumors (*2*). Later, specific genes were shown to influence both the spontaneous and induced tumors in the same manner (*3*, *4*, *5*).

The fact that the difference in degree of susceptibility, genetically established in the strain A and in the strain L lung tissue, persisted in large

part at least when the tissues of the two strains were surviving in a common host, indicates that the action of at least most of the susceptibility genes by which the two strains differ is confined to the lung tissue. It is to be assumed that all the genes would occur in the lung cells, but this would not necessarily limit the action of the susceptibility genes to the lung tissue. They, likewise, would occur in all other cells, excepting nonnucleated cells. Not all genes controlling mammary-tumor development in the mouse have their action limited to the mammary tissue. The action of some of these genes is manifested through the endocrine system.

In this experiment, however, there is a slight indication that a minor part of the genic influence on pulmonary-tumor development may be manifested through a general systemic mechanism. The LA F_1 host may have slightly reduced the susceptibility of the transplanted strain A lung tissue. From previous work (*1*) one could expect a strain A mouse injected intravenously with 0.5 mg. of dibenzanthracene for 12 to 15 months to have well over 100 tumor nodules in the lungs. Since each lung transplant in the present experiment consisted of approximately one thirtieth of the complete lungs of the strain A donor, one might have expected each of the strain A transplants to have had 3 or more tumor nodules. Only 39 percent of these transplants had nodules, however, and in only 1 transplant was there more than 1 nodule. This result suggests that either the maintaining of the transplant in the LA F_1 host, which is less susceptible than strain A, or the transplantation *per se* resulted in less tumors than might otherwise have been expected. The effect of transplantation *per se* is being investigated.

On the other hand, the LA F_1 hosts may have slightly increased the susceptibility of the strain L lung transplants. In previous work (*1*) only 24 percent of strain L mice injected intravenously with 0.5 mg. of dibenzanthracene developed pulmonary tumors, and in only 1 was there more than 1 nodule. That animal had 2 nodules. Considering that approximately 30 transplant fragments were cut from the lungs of each strain L donor, one might have expected even less than the 3 tumors observed in the strain L transplants. Furthermore, the tumors in the transplants from the strain L donors were larger than any ever observed in strain L mice, which offers additional evidence of a slight effect of the LA F_1 hosts that are more susceptible to the development of pulmonary tumors than are mice of strain L.

The lethal yellow gene, not involved in the study of these two strains, has been shown specifically to increase susceptibility to pulmonary tumors (*4*, *5*). Since it also has an influence upon the growth of the animals, it might be expected that its effect upon pulmonary-tumor development would be through some systemic action. It will be of interest to see how its effect is manifested in lung transplants in a situation similar to that reported here. It will also be interesting to make comparable studies in respect to other specific genes that have been shown to be related to susceptibility to pulmonary-tumor development.

SUMMARY

In order to ascertain whether genic action controlling the development of pulmonary tumors in mice is localized in the lung tissue or is manifested through some general systemic mechanism, lung tissues from strain A, which is highly susceptible to pulmonary tumors, and from strain L, which is highly resistant to pulmonary tumors, were transplanted subcutaneously into a common host, the F_1 hybrid of the two strains. Following the intravenous injection of dibenzanthracene into the hosts, pulmonary tumors were found in 39.1 percent of the transplants from strain A donors and in 3.6 percent of the transplants from strain L donors. Thus, a large part of the difference in genetic susceptibility to pulmonary tumors of the donor strains was retained in the transplanted tissues, suggesting that the action of at least most of the susceptibility genes by which these two strains differ is localized in the lung tissue.

Proliferation of the bronchial epithelium was often found in the microscopic sections of the transplanted lung fragments. The characteristics by which it was distinguished from the pulmonary tumors are given.

REFERENCES

(*1*) HESTON, W. E.: Genetic analysis of susceptibility to induced pulmonary tumors in mice. J. Nat. Cancer Inst. 3: 69–78, 1942.

(*2*) ———: Inheritance of susceptibility to spontaneous pulmonary tumors in mice. J. Nat. Cancer Inst. 3: 79–82, 1942.

(*3*) ———: Relationship between the lethal yellow (A^y) gene of the mouse and susceptibility to induced pulmonary tumors. J. Nat. Cancer Inst. 3: 303–308, 1942.

(*4*) HESTON, W. E., and DERINGER, M. K.: Relationship between the lethal yellow (A^y) gene of the mouse and susceptibility to spontaneous pulmonary tumors. J. Nat. Cancer Inst. 7: 463–465, 1947.

(*5*) ———: Relationship between the hairless gene and susceptibility to induced pulmonary tumors in mice. J. Nat. Cancer Inst. 10: 119–124, 1949.

(*6*) MORRIS, H. P.: Diet and some other environmental influences in the genesis and growth of mammary tumors in mice. Pub. Am. Assoc. Advancement Sc. 22: 140–161, 1945.

(*7*) NORRIS, R. F.: Pulmonary adenomatosis resembling jagziekte in the guinea pig. Arch. Path. 43: 553–558, 1947.

(*8*) PASSEY, R., LEESE, A., and KNOX, J.: Bronchiectasis and metaplasia in the lung of the laboratory rat. J. Path. Bact. 42: 425–434, 1936.

(*9*) LORENZ, E., and STEWART, H. L.: Tumors of the alimentary tract induced in mice by feeding olive-oil emulsions containing carcinogenic hydrocarbons. J. Nat. Cancer Inst. 7: 227–238, 1947.

(*10*) FEKETE, E.: Histology. *In* Staff of the Roscoe B. Jackson Memorial Laboratory, Biology of the Laboratory Mouse. Ch. 3. Blakiston Co., Philadelphia, 1941.

(*11*) DERINGER, M. K., and DUNN, T. B.: Mast-cell neoplasia in mice. J. Nat. Cancer Inst. 7: 289–298, 1947.

(*12*) ANDERVONT, H. B.: Pulmonary tumors in mice. VII. Further studies on the serial transmission of lung tumors occurring in inbred mice. Pub. Health Rep. 54: 1519–1524, 1939.

(*13*) SMITH, W. E.: The tissue transplant technic as a means of testing materials for carcinogenic action. Cancer Research 9: 712–723, 1949.

(*14*) HORNING, E. S.: Studies on the induction of lung cancer in mice. Brit. J. Cancer 4: 235–244, 1950.

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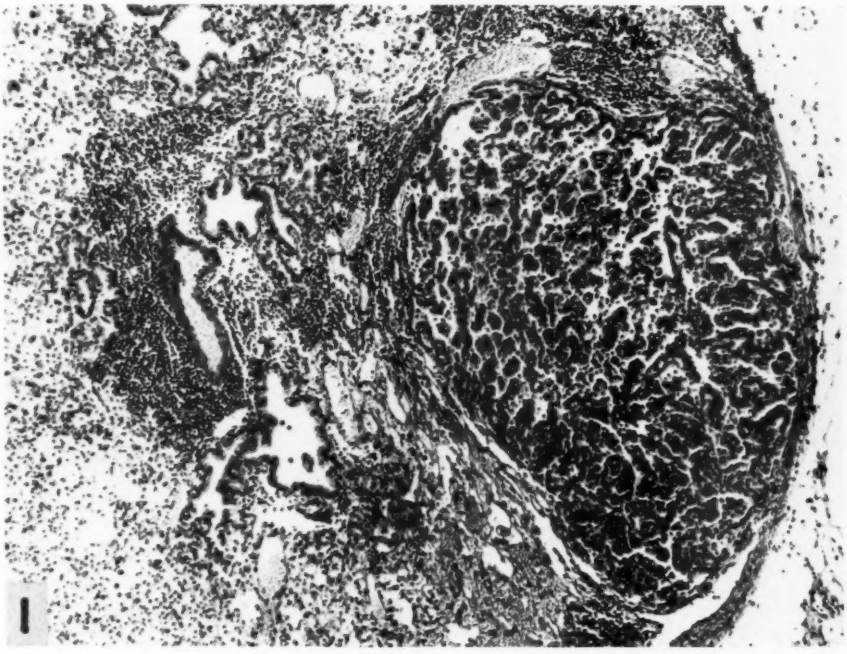
PLATE 114

Pulmonary tumors in fragments of lung from strain A mice transplanted to LA F_1 hybrids

FIGURE 1.—Note peripheral location of the tumor on right at a distance from the larger bronchi, and note typical histologic picture. Proliferation of the bronchial epithelium can be seen between the lung tumor and a bronchus. Hematoxylin and eosin stain. × 110.

FIGURE 2.—Note coalescing pulmonary tumors. Hematoxylin and eosin stain. × 110.



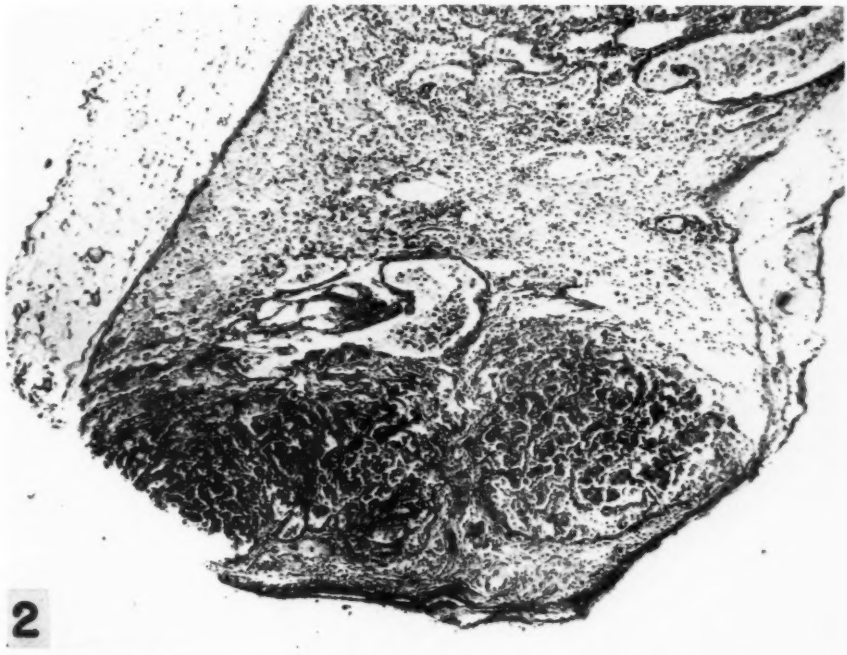


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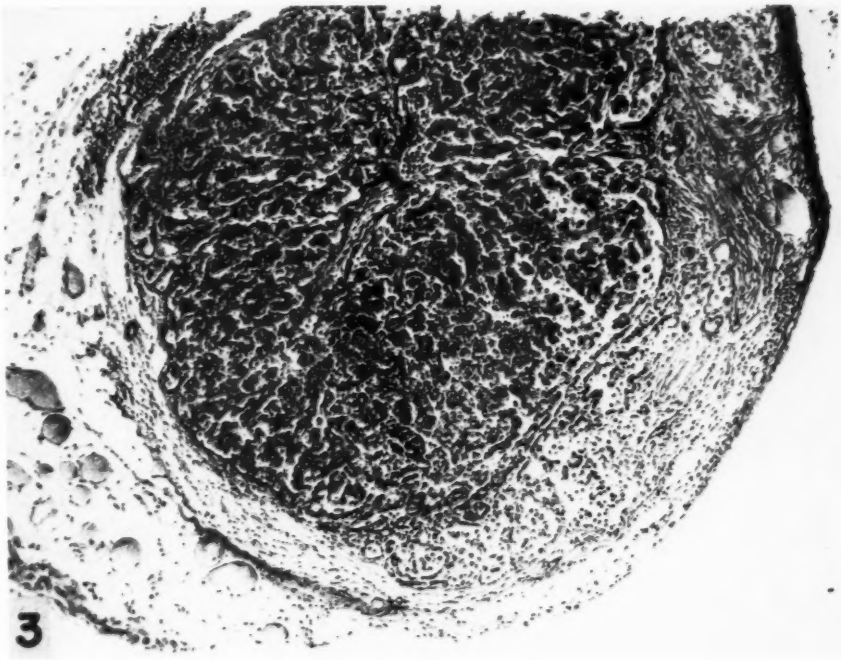
PLATE 115

Pulmonary tumors in fragments of lung from strain A mice transplanted to LA F_1 hybrids

FIGURE 3.—Pulmonary tumor showing evidence of progressive growth into the capsule where small spaces lined by epithelial cells are found. Hematoxylin and eosin stain. × 110.

FIGURE 4.—Comparatively large pulmonary tumor. Remaining lung tissue is notably free from inflammatory reaction. Hematoxylin and eosin stain. × 65.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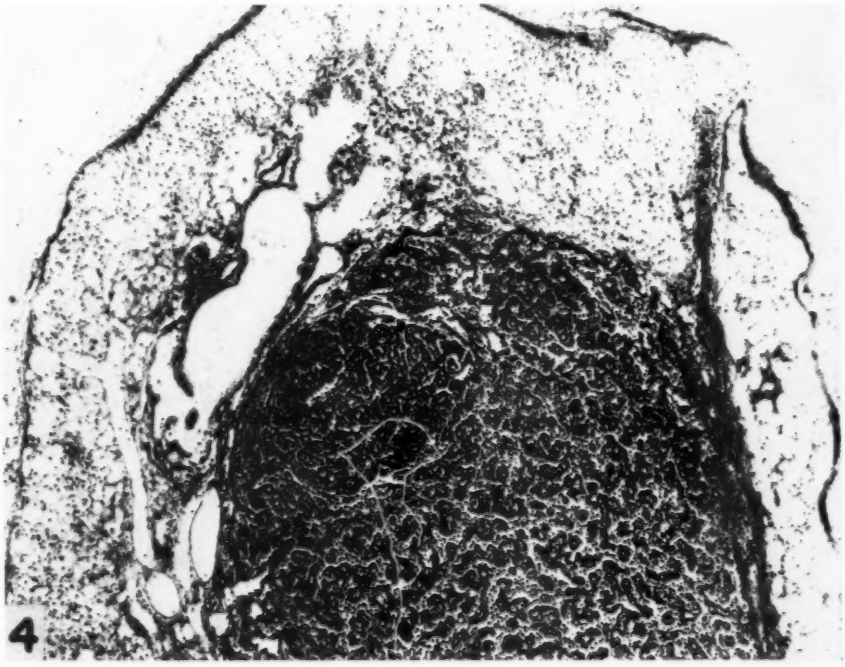
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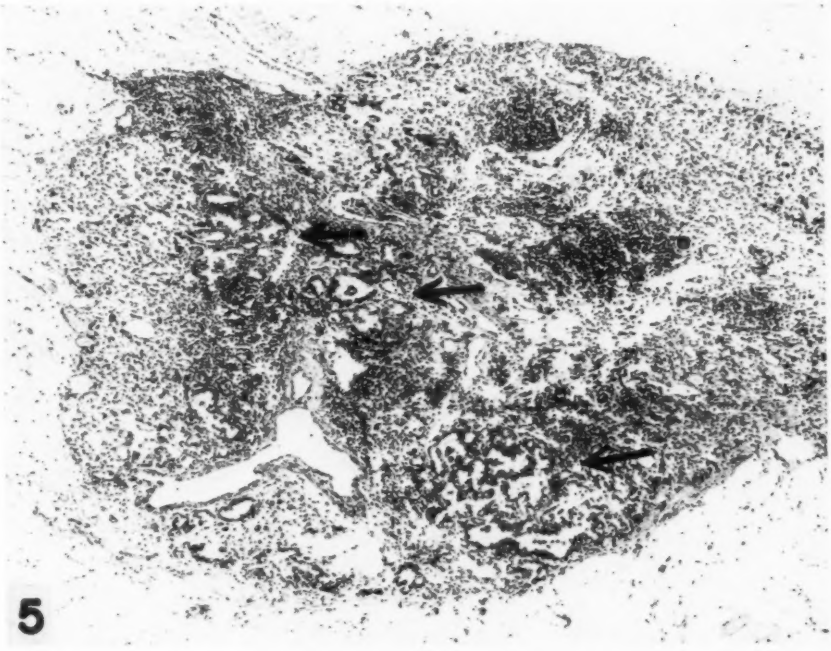
PLATE 116

Proliferation of bronchial epithelium in fragments of lung from strain L mice transplanted to LA F_1 hybrids

FIGURE 5.—Several foci of proliferation of bronchial epithelium marked by arrows. Note location in central portion and inflammatory reaction in adjacent tissue. Hematoxylin and eosin stain. × 90.

FIGURE 6.—Small space in an area of proliferation of the bronchial epithelium surrounded by ciliated epithelial cells. Hematoxylin and eosin stain. × 1,500.



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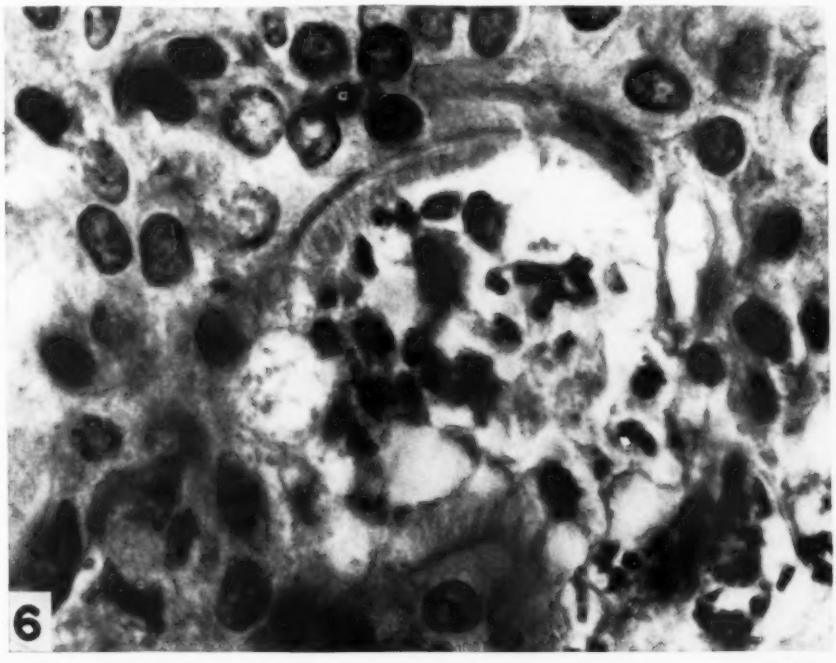

6

THE EFFECT OF SUPPLEMENTARY CHOLINE ON THE LIVERS OF RATS FED *p*-DIMETHYLAMINOAZOBENZENE[1,2]

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Supplements of choline and cystine were found by György, Poling, and Goldblatt (*1*) to have an inhibiting effect on tumor production by *p*-dimethylaminoazobenzene in a rice diet. Moreover, no tumors appeared during the experimental period of 175 days in any of their animals fed the dye incorporated in a 6- or 18-percent-casein diet supplemented with crystalline vitamin-B factors. White and Edwards (*2*) observed, however, that the addition of choline to a yeast-supplemented 6-percent-casein diet had no effect upon the final incidence of *p*-dimethylaminoazobenzene-induced liver tumors in rats.

It was also found by White and Edwards (*3*) that the cystine restriction of a *p*-dimethylaminoazobenzene-supplemented diet containing 6-percent casein resulted in a prolongation of the latent period and possibly a reduction in the total incidence of hepatic tumors when fed to rats *ad libitum*. When the paired-feeding technique was employed by White and White (*4*) cystine deprivation appeared not to lengthen the latent period and probably exerted no effect on the total production of hepatomas.

Other studies pertinent to the relation of the dietary restriction of sulfur-containing amino acids or of choline, or both, to the induction of hepatomas of the rat by *p*-dimethylaminoazobenzene have been reported by White (*5*) and by Jacobi and Baumann (*6*). Voegtlin, Johnson, and Thompson (*7*) and Morris and Voegtlin (*8*), earlier, investigated the effect of dietary restriction of glutathione and of methionine, respectively, on tumor growth.

The present investigation was carried out some years ago to determine whether or not choline might be a factor in the inhibiting effects which had been reported (*9–12*) for yeast, liver, rice polishings, and wheat upon the production of liver cancers in rats by *p*-dimethylaminoazobenzene. The study was discontinued after 180 days because of the war. The results, therefore, give no information on the final incidence of tumors. The data are reported because the feeding regimen was carefully followed,

[1] Received for publication February 20, 1950.

[2] This investigation was begun in the department of biochemistry of The George Washington University School of Medicine. The author is indebted to Dr. Thelma B. Dunn of The National Cancer Institute for the histologic report.

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the restricted dietary choline was shown to have a significant effect on the lipid content of the livers of the dye-fed animals, and liver lesions, including hepatomas, did appear during the period of observation.

EXPERIMENTAL REGIMEN

In the first series of rats three litters of 8 weanlings each (bred from Wistar-strain stock rats) were maintained for 6 months on a basal diet of (Labco) vitamin-free casein 18, sucrose 56, salt mixture (*13*) 4, agar 2, and Mazola oil 20 percent. Two dextrin pills containing per pill 10 micrograms each of thiamine, riboflavin, and pyridoxine, 40 micrograms of calcium pantothenate, and 125 micrograms of nicotinic acid, were administered daily.[3] 2-Methyl-1-4-naphthoquinone was incorporated in the diet at a concentration of 1 mg. per kilogram. One drop of a mixture of halibut-liver oil and Viosterol in Mazola oil, given each rat every fourth day, furnished an average daily allowance of 200 Int. units of vitamin A, and 20 Int. units of vitamin D. Thirteen animals were fed the diet without choline supplements, and the remaining 11 rats were given 20 mg. of choline chloride daily in the B-complex pills. Six rats in the choline-supplemented group and 7 with the unsupplemented basal diet had 0.06 percent *p*-dimethylaminoazobenzene incorporated in the food. Thus, 11 of the 24 rats in this series were maintained as controls to observe the effect of the choline supplements uncomplicated by the addition of the dye.

Diet No. 2, employed in a second series, differed from diet 1 only by the substitution of 9 percent of the casein by 9 percent of arachin, the arachin having been isolated from peanut flour by the method of Johns and Jones (*14*). It is estimated[4] that, per gram, diet 1 furnished approximately 0.65 mg. of cystine and 6.3 mg. of methionine, while diet 2 provided 1.22 mg. of cystine and 3.7 mg. of methionine.

Twenty rats from 4 litters were used in this second series and they were divided into 2 groups, one of 4 control rats without *p*-dimethylaminoazobenzene and the other of 16 rats with 0.06 percent of the dye in the basal diet. Two of the control rats and 8 of the dye-supplemented animals received choline supplements.

The animals were kept in individual metabolism cages with raised large-mesh wire-floor screens and they were given the diets *ad libitum*. Records were made twice weekly of food consumption, weight, and the appearance of each rat. Analyses for choline were made of the individual constituents of the diet and of the whole mixed diet by the colorimetric method of Beattie (*16*) for choline reineckate, and, after acetylation, on strips of rat intestine according to the method of Fletcher, Best, and Solandt (*17*) for

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[3] We wish to express appreciation to Merck and Co., Inc., for their generous supply of choline chloride, and for the crystalline vitamin-B factors, and to the Traders Oil Mill Company of Fort Worth, Texas, for their kindness in furnishing us with peanut flour (McMath-Howard Process PDP).

[4] Calculations for casein based on an average cystine content of 0.36 ± 0.04 percent and an average methionine content of 3.5 ± 0.3 percent, and for arachin 1.0 ± 0.2 percent cystine and 0.6 ± 0.1 percent methionine, as reported by Block and Bolling (*15*).

acetylcholine. Both methods indicated that the diet contained no more than a trace of choline (<0.03 mg. per gm.).

After 6 months the rats were killed. A small portion (approximately 0.5 gm.) of each liver of the dye-fed animals, taken from a nodule when nodules were present, was preserved in 10 percent formalin for histologic examination. The remainder of each of these livers and all of each control liver were analyzed for fatty acids and total cholesterol by slightly modified methods of Stoddard and Drury (*18*) and Schoenheimer and Sperry (*19*), respectively.

RESULTS

Lipid content of livers.—Evidence of an effect of the choline supplements with these diets is found in the chemical data (table 1, columns 7 and 8) which demonstrate significantly lower fatty acid and cholesterol contents of the livers of the choline-supplemented animals than of those of the restricted litter mates. This finding is consistent with the lipotropic effect of choline first reported by Best and co-workers (*20*). The chemical analyses, however, represent the fatty acid and cholesterol concentrations of liver plus nodular material and carcinomas when they were present. In many cases in the choline-fed rats there was little normal appearing liver tissue present. Kishi, Fijiwara, and Nakahara (*21*) reported that hepatoma contained about one-third the amount of total fatty acids found in normal liver. It is possible, therefore, except in the case of basal diet 1, with no dye, that the lower concentration of fatty acid found in the livers of the choline-supplemented animals may have been due, in part, to the presence of a larger amount of cancer or precancerous tissue. The livers that had the higher content of fatty acid were those that showed histologically a high degree of fatty infiltration.

Since the livers of control animals that received the diets without the carcinogen were normal in appearance, except for a slightly yellowish color in those of rats on the choline-low diets, histologic examination of them was omitted.

Macroscopic observations on livers of p-dimethylaminoazobenzene-fed animals.—At necropsy the livers of the rats fed choline supplements contained large numbers of firm, whitish nodules, while the livers of the litter mates not fed choline had few nodules (table 2). The nodules in some livers were small (1 to 2 mm. in diameter), numerous, and regularly distributed throughout one or more lobes, and in others varied in diameter up to 2 cm. and protruded beyond the liver boundaries. In table 2 is indicated the comparative extent of involvement of each liver with respect to these nodules.

In no case was cirrhosis of the liver observed macroscopically.

Histologic observations on liver of p-dimethylaminoazobenzene-fed animals.—The livers of the rats receiving *p*-dimethylaminoazobenzene showed the same histologic features that have been described by Edwards and White (*22*) and by Opie (*23*). The lesions of most significance have been listed in table 2. These were:

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TABLE 1.—*Summary of dietary and analytical data*

Diet	No. of rats	Average daily consumption		Average daily gain in wt.		Average content of fatty acids in livers [1] (cc. 1.0 N NaOH/100 gm. of fresh liver)	Average content of cholesterol in livers (mg./100 gm. of fresh liver)	Average weights of livers			
		Food	Carcinogen					Total gm.		Percent of body weight	
		gm.	*mg.*	*gm.*	*gm./gm. food*			*Female*	*Male*	*Female*	*Male*
Basal diet No. 1,[2] no choline	6	9.4		1.00	0.10	21.8 (11.4–33.3)	540	7.6	8.7	3.7	4.0
Basal diet No. 1, with choline	5	10.2		1.05	0.10	8.8 (6.1–13.2)	305	4.9	9.1	2.6	3.4
p-Dimethylaminoazobenzene diet No. 1, no choline	7	8.4	5.0	.80	0.095	31.5 (15.0–54.3)	637	7.4	12.9	5.2	6.3
p-Dimethylaminoazobenzene diet No. 1, with choline	6	8.8	5.3	.90	0.10	11.5 (8.7–13.2)	440	9.9	16.7	5.7	7.5
Basal diet No. 2,[3] no choline	2	9.5		.69	0.07	[4] 11.8	228	6.9		2.9	
Basal diet, No. 2, with choline	2	10.2		1.30	0.13	11.7 (7.3 and 16.1)	274	6.9	11.4	2.9	3.3
p-Dimethylaminoazobenzene diet No. 2, no choline	7	10.8	6.5	.77	0.07	49.8 (27.9–69.2)	697	13.7	13.7	8.4	7.6
p-Dimethylaminoazobenzene diet No. 2, with choline	8	9.2	5.5	1.00	0.11	11.4 (7.9–17.1)	449	15.9	17.7	8.7	8.1

[1] Assuming 277 to be the molecular weight of an average fatty acid, the fatty acids obtained from the livers varied from 1.7 to 4.7 percent in the choline supplemented animals and from 3.2 to 19.2 percent in the animals not fed choline.

[2] Diet No. 1: 18 percent casein in the diet.

[3] Diet No. 2: 9 percent casein and 9 percent arachin in the diet.

[4] Only 1 liver analyzed.

1. Fatty deposit in the cytoplasm of the parenchymal cells. In extreme cases, the cytoplasm of the liver cells was uniformly crowded with fat globules, which pushed the nucleus to one side.

2. Cirrhosis. This was recognized microscopically by the formation of fibrous tissue that separated groups of parenchymal liver cells. The

TABLE 2.—*Morphological changes in livers of rats fed p-dimethylaminoazobenzene*

Rat				Morphologic changes in livers (histologic)	
No. and sex	Litter	Diet	Macroscopic (nodules [1])	Neoplastic and other changes [2]	Degree of fatty deposit [3]
				(No choline supplements)	
2 ♀	I	1	one, 1 mm.	(−)	++
3 ♂	I	1	0, cysts	B. D. P., cystic areas	++++
11 ♀	II	1	++	B. D. P	+++
12 ♂	II	1	++	B. D. P., cystic areas	++++
20 ♀	III	1	++	B. D. P	++++
19 ♂	III	1	+	B. D. P	++++
21 ♂	III	1	++	B. D. P	++++
43 ♀	V	2	+	*Carcinoma*, B. D. P., cirrhosis	++++
42 ♂	V	2	0	Several hyperplastic nodules, cirrhosis	++++
50 ♀	VI	2	+	B. D. P., cirrhosis	++++
51 ♂	VI	2	0	Cirrhosis	++++
53 ♂	VI	2	++	Hyperplastic nodules, cirrhosis	++++
56 ♀	VII	2	+	B. D. P., cirrhosis	+++
57 ♂	VII	2	0	(−)	+
				(With choline supplements)	
7 ♀	I	1	++	No significant lesion	0
8 ♂	I	1	++	No significant lesion	0
15 ♀	II	1	+++++	*Carcinoma*	0
16 ♂	II	1	+++++	*Carcinoma*, B. D. P	0
24 ♂	III	1	+++++	Extensive B. D. P	0
25 ♂	III	1	+++++	*Carcinoma*, B. D. P	0
44 ♂	V	2	++	*Carcinoma*, B. D. P., cirrhosis	+
46 ♀	VI	2	+++++	Sections missing	
48 ♀	VI	2	+++++	B. D. P	+
45 ♂	VI	2	0	Cirrhosis	+
47 ♂	VI	2	++	No significant lesion	±
49 ♂	VI	2	+++++	*Carcinoma*, B. D. P	+
54 ♀	VII	2	+++++	*Carcinoma*, B. D. P	0
55 ♂	VII	2	+++++	B. D. P., cirrhosis	+

[1] Nodules: +=few in one lobe; ++=moderate number in two lobes; +++++=liver filled with nodules.
[2] B. D. P.: bile duct proliferation.
[3] Degree of fatty deposit: ±=trace; +, ++, +++, ++++=relative numbers of areas involved.

cirrhosis in these animals was generally mild, and it was not observed macroscopically.

3. Cystic areas. These were collections of multiple small cysts lined by a single layer of flat or cuboidal epithelium.

4. Bile duct proliferation. This lesion was particularly studied by Edwards and White. Small circular and tubular spaces lined by a single layer of cuboidal epithelium were separated by varying amounts of new-

formed connective tissue. Where such lesions reached the capsule, the surface was depressed, and there was no suggestion of increasing bulk or neoplastic growth. The process varied considerably in extent, in some cases involving only the portal areas, and in other cases replacing large areas of liver parenchyma.

5. Hyperplastic nodules. These were small areas composed of liver cells that were larger in size and often more heavily stained than surrounding normal cells. They represented either early hepatomas or areas of regenerating liver tissue. They differed from frank carcinoma in the small area involved and relatively normal appearance of the cells.

6. Carcinoma. This term was used to include all neoplastic processes whether of liver-cell or bile-duct-cell type. The majority were hepatomas composed of solid groups of cells resembling liver cells, but others formed glandular structures.

When these lesions were tabulated for the 2 groups (table 2) it was found that fatty deposits in the liver cells were more frequent in animals without the choline supplement. Only 1 carcinoma occurred in this group of 14 rats, however, while 6 were found in the group of 13 rats with the choline supplement.

Bile-duct proliferation was observed as frequently in the livers of the noncholine-supplemented rats as in those of the choline-supplemented animals with both diets.

Histologically, liver cirrhosis was found in 9 of the rats on the casein-arachin diet (No. 2), and twice as frequently in those without choline as in those receiving choline supplements with this diet. No cirrhosis was observed in the livers of rats receiving the casein diet (No. 1).

SUMMARY

Six months after the beginning of the experimental period, during which rats were maintained on choline-restricted diets containing *p*-dimethylaminoazobenzene and 18 percent casein, or 9 percent casein plus 9 percent arachin, the livers of animals that received choline supplements contained approximately one-third the amount of fatty acids and two-thirds of the cholesterol of those of nonsupplemented litter mates.

Macroscopically, striking differences were observed between the livers of the animals with and without supplementary choline. The livers of the choline-supplemented rats were enlarged, and they contained numerous firm, white nodules, usually involving the entire organ. Histologically, bile-duct proliferation was observed as frequently in the livers of the choline-restricted rats as in those receiving supplementary choline.

Carcinoma of the liver was present in 1 of 14 rats that received the basal diet only and in 6 of 13 litter mates that received the basal diets with supplements of choline. This difference in the numbers of livers having carcinomas after 180 days with and without dietary choline cannot be interpreted as indicating the possibility of an effect of choline restriction upon

the final incidence of hepatomas. The appearance of carcinoma in 1 liver of the 14 rats on the choline-restricted diet shows that tumors can occur under the dietary conditions without choline supplements.

Although the number of animals is small, the results suggest that the period of induction of liver tumors by *p*-dimethylaminoazobenzene is longer in the rats maintained on the restricted diets than in those receiving the choline supplements.

REFERENCES

(*1*) GYÖRGY, P., POLLING, E. C., and GOLDBLATT, H.: Necrosis, cirrhosis, and cancer of liver in rats fed a diet containing dimethylaminoazobenzene. Proc. Soc. Biol. Med. 47: 41–44, 1941.

(*2*) WHITE, J., and EDWARDS, J. E.: Effect of supplementary methionine or choline plus cystine on the incidence of *p*-dimethylaminoazobenzene-induced hepatic tumors in the rat. J. Nat. Cancer Inst. 3: 43–59, 1942.

(*3*) ———: Effect of dietary cystine on the development of hepatic tumors in rats fed *p*-dimethylaminoazobenzene. J. Nat. Cancer Inst. 2: 535–538, 1942.

(*4*) WHITE, F. R., and WHITE, J.: Effect of cystine per se on the formation of hepatomas in rats (following the ingestion of *p*-dimethylaminoazobenzene. J. Nat. Cancer Inst. 7: 99–101, 1946.

(*5*) WHITE, J.: Retardation of growth of the rat ingesting *p*-dimethylaminoazobenzene (butter yellow). I. The effect of various dietary supplements. J. Nat. Cancer Inst. 1: 337–341, 1940.

(*6*) JACOBI, H. P., and BAUMANN, C. A.: Choline in tumor-bearing animals and a choline-like effect of butter yellow. Cancer Research 2: 175–180, 1942.

(*7*) VOEGTLIN, C., JOHNSON, J. M., and THOMPSON, J. W.: Glutathione and malignant growth. Pub. Health Rep. 51: 1689–1697, 1936.

(*8*) MORRIS, H. P., and VOEGTLIN, C.: The effect of methionine on normal and tumor growth, J. Biol. Chem. Proc. 133: lxix, 1950.

(*9*) NAKAHARA, W., FUJIWARA, T., and MORI, K.: Inhibiting effect of yeast feeding on the experimental production of liver cancer. "Gann" 33: 57–65, 1939.

(*10*) KINOSITA, R.: Studies on carcerogenic chemical substances. J. Jap. Soc. Diseases Digestive Organs 37: 513–592, 1938.

(*11*) ANDO, T.: Experimentelle Leberkarzinomentstehung und Getreide. I. Mitteilung. "Gann" 32: 252–257, 1938.

(*12*) NAKAHARA, W., MORI, K., and FUJIWARA, T.: Effect of diets on the experimental production of liver cancer. "Gann" 33: 406–427, 1939.

(*13*) OSBORNE, T. B., and MENDEL, L. B.: The nutritive value of the wheat kernel and its milling products. J. Biol. Chem. 37: 572, 1919.

(*14*) JOHNS, C. O., and JONES, D. B.: The proteins of the peanut, *Arachis hypogaea*. I. The globulins arachin and conarachin. J. Biol. Chem. 28: 77–87, 1916–17.

(*15*) BLOCK, R. J., and BOLLING, D.: The amino acid composition of proteins and foods. Charles C. Thomas, Springfield, Ill., 1945.

(*16*) BEATTIE, F. J. R.: A colorimetric method for the determination of choline and acetylcholine in small amounts. Biochem. J. 30: 1554–1559, 1936.

(*17*) FLETCHER, J. P., BEST, C. H., and SOLANDT, O. McK.: The distribution of choline. Biochem. J. 29: 2278–2284, 1935.

(*18*) STODDARD, J. L., and DRURY, P. E. A titration method for blood fat. J. Biol. Chem. 84: 741–748, 1929.

(*19*) SCHOENHEIMER, R., and SPERRY, W. M.: A micromethod for the determination of free and combined cholesterol. J. Biol. Chem. 106: 745–760, 1934.

(*20*) BEST, C. H., and CHANNON, H. J.: The action of choline and other substances in the prevention and cure of fatty livers. Biochem. J. 29: 2651–2658, 1935.

(21) KISHI, S., FUJIWARA, T., and NAKAHARA, W.: Comparison of chemical composition between hepatoma and normal liver tissues. VIII. Lipoids, "Gann" 33: 332–340, 1939.

(22) EDWARDS, J. E., and WHITE, J.: Pathologic changes with special reference to pigmentation and classification of hepatic tumors in rats fed *p*-dimethylaminoazobenzene (butter yellow). J. Nat. Cancer Inst. 2: 157–183, 1941.

(23) OPIE, E. L.: The pathogenesis of tumors of the liver produced by butter yellow. J. Exper. Med. 80: 231–246, 1944.

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